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(54) Title: NOVEL PEPTIDES AND METHOD FOR ALTERING THE ACTIVITY OF ALLOSTERIC PROTEINS

(57) Abstract

A method for rapidly producing effector peptides that alter a functional activity of an allosteric protein uses a target region in the amino acid sequence encoding the protein. The peptides are substantially identical in sequence to portions of the target region. A method of altering the functional activity of an allosteric protein depends on an interaction of these effector peptides derived from the protein itself with the protein. The method is capable of either increasing or decreasing the activity of the protein. The method is particularly applicable to human epidermal growth factor receptor; peptides that can either inhibit or activate the protein tyrosine kinase activity of the human growth factor receptor are part of the invention.

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NOVEL PEPTIDES AND METHOD FOR ALTERING THE ACTIVITY OF ALLOSTERIC PROTEINS

FIELD OF THE INVENTION

This invention relates to novel peptides and a method for altering the biological activity of allosteric proteins, and more particularly to a method for inhibiting or activating allosteric proteins using effector peptides and to the peptides obtained.

BACKGROUND OF THE INVENTION

Allosteric proteins may be characterized as having flexibility in structure so that interaction of the protein with an allosteric effector molecule at one site affects the three-dimensional conformation of the protein and thus its interactions with other molecules at one or more additional site(s). The other molecules may be substrate molecules or ions, or other sites on the allosteric protein involved in intermolecular interactions. The biological activity of a given allosteric protein, for example enzymatic activity, is a function of precise structural relationships between regions of the protein. Thus, certain structural ("allosteric") transitions induced by allosteric effectors and facilitating interactions between regions of the allosteric protein molecule are required to achieve a three-dimensional structure that supports expression of functional activity of the protein, e.g. for the protein to bind a substrate molecule or ion, or support inhibition of functional activity.

Many familiar proteins, such as hemoglobin, the oxygen-carrying protein in blood, are allosteric. Allosteric proteins that have enzymatic activity are involved in many important physiological processes in man. For example, allosteric enzymes include receptors that

behave as protein kinases. The activity of protein kinases is considered critical in the regulation of cellular functions including regulation of metabolism, cell growth and differentiation. A number of protein kinases have been shown to be highly elevated in particular types of human cancer. It is believed that many cases of cancer arise from the abnormal activity of altered cellular genes, known as oncogenes. These genes, or the amount of expression of their gene products, can be altered as the result of infection by oncogenic viruses or chemical damage by carcinogens. For example, the receptor for epidermal growth factor (EGF), an oncogene encoded protein that has tyrosine kinase activity, has been shown to be highly elevated in a serious form of cancer known as squamous cell carcinoma (Kamata et al., Cancer Res. 46:1648-1653 (1986); Cowley et al., Br. J. Cancer 53:223-229 (1986) and Filmus et al., Biochem. Biophys. Res. Commun. 128:898-905 (1985)). A receptor kinase, homologous to EGF receptor in the intracellular domain, and designated HER-2 has been shown to be overexpressed in other cancers, including certain forms of breast cancer (Slamon et al., Science 235:177-182 (1987)). The Abelson oncogene encodes a protein kinase associated with certain leukemias (Rosenberg et al., Adv. Virus Res., 35:39-81 (1988)).

Aspartate transcarbamoylase (ATCase) from E. coli is an allosteric protein that has been used as a model for study of both homotropic (interactions within a subunit of the protein) and heterotropic (interactions between subunits) in proteins (See, Kantrowitz and Lipscomb, TIBS 15:53-59 (1990)). In this protein, contacts are made and/or broken between portions of the polypeptide chain(s) as the protein changes from active to inactive state, or vice versa. The making and breaking of the intramolecular interactions between specific residues in ATCase occur between series of amino acid residues, each on relatively short (less than 12 amino acids) opposing sequences on the

enzyme (Id.)

However, ATCase is one of only a very few proteins for which detailed high resolution structural information on allosteric interactions is available to define the consequences of activity-modifying ligands on structure. Moreover, extremely detailed x-ray crystallographic and other analytical studies have been performed on species of ATCase mutants modified by specific mutagenesis of the ATCase gene to verify interactions that are functionally important. These studies provide independent experimental evidence of the identity of the short interactive peptide sequences required to support activity of this allosteric protein.

Once a ligand, such as a peptide capable of altering the activity of an allosteric protein has been identified, many new forms of treatment for human disease or dysfunction associated with the activity of that protein may be developed. Thus, the platelet-derived growth factor (PDGF) receptor which is implicated in the formation of atherosclerotic lesions can be inhibited to prevent intralumenal smooth muscle cell migration that results in such lesions. Interleukin receptors such as the IL-1 receptor can be inhibited to control inflammation. The T-cell receptor involved in the mechanism of rejection of tissue transplants and pathogenic self-reactivity, may be selectively deactivated to prevent rejection of tissue transplants or suppress the self-reactivity associated with a variety of conditions including rheumatoid arthritis, allergic encephalitis, Hashimoto's thyroiditis, myasthenia gravis and other autoimmune diseases.

In addition, other human diseases or dysfunctions can be ameliorated by activation of certain allosteric proteins. For example, the insulin receptor may be activated to treat diabetes by enabling the body to use a

substitute for insulin that might be delivered orally. The epidermal growth factor (EGF) receptor can be activated to control ulcers and speed healing of corneal wounds and the receptor for PDGF and for other growth factors involved in wound healing can be activated to promote wound healing. Fibroblast growth factor (FGF) receptor may be activated to promote endothelial cell proliferation and the T-cell receptor complex can be activated to promote cellular immunity, resist infection and counter immunity-attacking conditions such as AIDS.

The involvement of allosteric proteins in a variety of human biological processes establishes a need for a simple, direct method for identifying lead compounds for development of ligand inhibitors for decreasing abnormally high activity of allosteric proteins and ligand activators for increasing abnormally low activity of allosteric proteins. Such "effector" ligands can provide for new approaches to disease intervention and provide useful adjuncts to present methods of therapy. Use of such ligands could allow lower doses of potentially toxic therapeutic agents to be used while avoiding side effects.

Previous approaches to developing ligands for modification of the activities of proteins have been time-consuming, labor-intensive and expensive. These approaches include extensive screening of organic chemicals, fermentation products, plant extracts and other sources of chemical compounds. Alternatively, known substrates or compounds known to bind to proteins and known to activate or inhibit their activity have been modified or improved (Yaish et al., Science 242:933-935 (1988)).

In addition, attempts have been made to use the primary amino acid sequence of proteins to modify activity of the protein. In these procedures, knowledge of the primary amino acid sequence of the protein is used in

conjunction with various procedures to elucidate regions of the amino acid sequence correlating with functions such as substrate binding or enzyme active sites. Thus, labeled substrate analogs have been used to react with a protein followed by peptide mapping to determine regions of sequence covalently bound to the substrate analogs to identify substrate binding sites. Site specific or "point" mutations have also been used to determine the effects of changes in specific amino acids on enzymatic activity or substrate binding. More sophisticated techniques include x-ray crystallographic analysis and molecular modeling using commercially available computer graphics systems to display and analyze the three-dimensional structure of a protein. In preparation for analysis by x-ray crystallography the protein may be crystallized together with substrate molecules or effector ligands so that the structure of the complex formed between the protein and the substrate sites at which the substrate or ligand which binds to it can be determined. Knowledge of the structure of ligand binding sites revealed by such analyses can facilitate synthesis of derivatives of the binding ligand with preferred pharmacological properties.

Once a type of protein has been characterized in this manner, it is possible to identify similar regions of amino acid sequence likely to be involved in similar functions for closely related or "homologous" proteins sharing common sequences. In addition, regions of amino acid sequence having hydrophobicity or hydrophilicity may be identified or predicted using hydropathy analysis (Kyte and Doolittle, J. Mol. Biol. 157:105 (1982)).

The information gathered from these procedures may be used to synthesize small peptides having sequences that correspond to the sites in the proteins identified as having a binding or structural function. For example, peptides derived from the binding sites on antigens that

recognize antibody have been used to examine antibody/antigen interactions (Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985)). Pierschbacher and Ruoslahti (Nature 309:30-33 (1984)) have synthesized small peptides (four amino acids in length) having cell attachment-inhibiting activity and having the same sequence as a cellular recognition sequence on fibronectin, an extracellular glycoprotein involved in cell attachment. These researchers used sequential proteolytic fragmentation to isolate a domain within the fibronectin molecule that inhibits cell attachment.

Synthetic peptides have also been prepared having inhibitory effects on protein kinases. These peptides typically compete with a substrate of the kinase for binding. For example, Yaish et al. (Science 242:933-935 (1988)) synthesized low molecular weight protein tyrosine kinase inhibitors with affinity for the substrate binding site of the EGF receptor kinase domain. The compounds were designed using, as a starting point, the structure of erbstatin, a compound which inhibits the autophosphorylation of the EGF receptor, and which competes with the phosphate acceptor substrate for binding to EGF receptor kinase and not with ATP. The compounds inhibited EGF receptor kinase activity as measured by EGF-dependent autophosphorylation of the receptor. Smith et al., (J. Biol. Chem. 265(4):1837-1840 (1990)) have described properties of inhibitory peptides that correspond to previously identified autoinhibitory domains of several kinases including calcium/calmodulin-dependent protein kinase II, smooth muscle myosin light chain kinase, protein kinase C and the heat-stable inhibitor of cAMP-dependent protein kinase (PKI-tide). Autoinhibitory domains of protein kinases are believed to inhibit kinase activity by interacting with elements of the catalytic domain including the substrate-binding site. Synthetic peptide analogs of autoinhibitory domains have been shown to inhibit kinase

activity competitively with respect to protein substrate. The sequences of autoinhibitory domains of protein kinases often contain basic amino acid residues resembling the natural substrate recognition sequence for kinase, but lacking a phosphate acceptor site, and are thought to be important for interactions with the catalytic domains as "pseudosubstrates" (Hardie, Nature 335:592-593 (1988)). Because many protein kinases share some of the same basic amino acid determinants for substrate recognition, Smith et al. hypothesized that synthetic peptides based on these "pseudosubstrate" sequences might be recognized by other kinases. They therefore examined the specificities of inhibition by these peptides and compared them with a peptide analog of the pseudosubstrate sequence of PKI-tide. The results demonstrated that peptides based on the regulatory domains of protein kinase C, calcium/calmodulin-dependent protein kinase II, and smooth muscle myosin light chain kinase were not specific inhibitors of their corresponding protein kinases. However, PKI-tide specifically inhibited cAMP-dependent protein kinase. This indicates that inhibitors or activators with structure based on the sequence of certain domains of a protein may be highly specific, acting only on proteins highly homologous with the sequence of that domain.

These studies provide examples of the prior art methods for identifying inhibitory ligands for modifying protein activity, but exhibit several disadvantages. These methods do not ordinarily allow the production of useful effectors unless a lead compound has already been identified or knowledge of high resolution three-dimensional structure of the allosteric protein or of its analogs or derivatives, is available, typically provided by X-ray crystallographic techniques. Moreover, such approaches are generally limited to known sites of intra- or intermolecular protein interactions or ligand-binding including sites of interaction between subunits. For

receptor proteins located on the surface of cells, three-dimensional structure information is largely unavailable because of the difficulty of crystallizing membrane proteins.

A method for the development of effector allosteric modifiers or "ligands" capable of modifying the functional activity of allosteric proteins, without requiring prior knowledge of a substantial amount of the three-dimensional structure of the protein or of specific ligand binding sites and structural relationships, would greatly facilitate the development of therapeutic agents and therapies for human diseases and dysfunctions.

SUMMARY OF THE INVENTION

A method for producing effector ligands which alter the functional activity of allosteric proteins, according to the present invention, meets this need. The method is based on the totally unexpected discovery that effector peptides corresponding to portions of the amino acid sequence (the "target sequence") of an allosteric protein can alter the activity of the allosteric protein. These effector peptides can either activate or inhibit the functional activity of the protein.

The method for producing effector peptides that alter a functional activity of an allosteric protein includes: 1) determining a target sequence of the primary amino acid sequence of an allosteric protein, containing at least one site of intramolecular or intermolecular contact within the allosteric protein, the site involved in an allosteric transition resulting in alteration of the expression of a functional activity of the allosteric protein; 2) synthesizing screening peptides of from about 10 to about 20 amino acids in length each of which is substantially identical to a region of the target sequence

and which in linear array correspond to substantially all of the target sequence of the primary amino acid sequence determined in step 1); and measuring a functional activity of the allosteric protein when reacted with each peptide to identify effector peptides that inhibit or activate a functional activity of the allosteric protein.

The invention also provides methods of using the effector peptides of the invention to alter the functional activity of an allosteric protein.

The effector peptide is at least 3 amino acids in length. The region of the amino acid sequence of the allosteric protein selected for synthesis of the peptides can contain amino acids capable of forming α-helical or β-pleated sheet secondary structure within the selected portion, and the peptide can then be identical or substantially identical to those amino acids capable of forming such ordered secondary structures.

The activity of the allosteric protein can be either increased or decreased by reaction with the effector peptides. This activity can be, for example, enzymatic activity or the binding affinity of the protein for a ligand.

An important illustration of this general method is its application to modification of the protein tyrosine kinase activity of the epidermal growth factor receptor (EGF). The process of the present invention can be used to either inhibit or stimulate this enzymatic activity.

The allosteric protein can be a receptor protein, enzyme, transport protein, nucleic acid binding protein and extracellular matrix protein. If a receptor protein, the allosteric protein can be epidermal growth factor receptor, insulin receptor, platelet-derived growth factor receptor,

tumor necrosis factor receptor, fibroblast growth factor receptor, erythropoietin receptor, lymphokine receptor and cytokine receptor.

The invention includes methods of using the effector peptides of the invention to alter a functional activity of an allosteric protein by reacting the allosteric protein with one or more of the effector peptides.

Another aspect of this invention is the effector peptides obtained that are capable of altering the expression of activity of an allosteric protein. These peptides are at least 3 amino acids in length, and preferably are at least 6 amino acids in length and capable of substantially altering, by inhibiting or activating, the expression of functional activity of an allosteric protein when reacted with the allosteric protein. Each peptide is substantially identical to a portion of a selected target region of the primary amino acid sequence of an allosteric protein, and when taken together in linear array correspond to substantially all of the target sequence. The target sequence contains at least one site of intramolecular or intermolecular contact within the allosteric protein, the site involved in an allosteric transition resulting in an alteration of the expression of functional activity of the allosteric protein. The effector peptides cause inhibition or activation of the functional activity of the allosteric protein when reacted with the protein.

Effector peptides of the invention for inhibiting tyrosine kinase activity of human epidermal growth factor receptor are as follows (SEQ ID NO:6): V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, T-V-Q-L-I-T-Q-L-M-P, W-C-V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, V-K-I-T-D-F-G-L-A-K-L-L-G, M-A-L-E-S-I-L-H-R-I-Y-T, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, P-A-S-E-I-S-S-I-L-E-K, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, W-M-I-D-A-

D-S-R-P-K-F, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, F-Y-R-A-L-M-D-E-E-D-M-D, D-D-V-V-D-A-D-E-Y-L-I-P, D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, A-K-L-L-G-A-E-E-K-E-Y-H-A-E-G, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, V-Q-I-A-K-G-M-N-Y-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, I-M-V-K-C-W-M-I-D-A-D, P-L-T-P-S-G-E-A-P, Y-L-V-I-Q-G-D or D-E-Y-L-I-P-Q-Q-G-F-F for reacting with the region between amino acids 646 to 1015 in the amino acid sequence encoding the EGF receptor, when the peptide is present at a concentration of about 1 mM, and cause at least 34% inhibition of the tyrosine kinase activity of human epidermal growth factor.

Of these peptides, more preferred are the following peptides, which cause at least 50% inhibition of the tyrosine kinase activity of human epidermal growth factor when the peptide is present at a concentration of about 1 mM (SEQ ID NO:6): T-V-Q-L-I-T-Q-L-M-P, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, I-M-V-K-C-W-M-I-D-A-D, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, D-D-V-V-D-A-D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, Y-L-V-I-Q-G-D, D-E-Y-L-I-P-Q-Q-G-F-F.

Even more preferred inhibitory peptides are the following peptides that cause at least 75% inhibition of the tyrosine kinase activity of human epidermal growth factor when the peptide is present at a concentration of about 1 mM (SEQ ID NO:6): K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L,A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D or I-M-V-K-C-W-M-I-D-A-D.

Most preferred inhibitory peptides are (SEQ ID

NO:6): A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D or V-Q-I-A-K-G-M-N-Y-L that cause at least 85% inhibition of the tyrosine kinase activity of human epidermal growth factor when the peptide is present at a concentration of about 1 mM.

The invention also provides effector peptides for stimulating activity of an allosteric protein. Effector peptides for stimulating the tyrosine kinase activity of human epidermal growth factor receptor having the amino acid sequence (SEQ ID NO:6) R-R-H-I-V-R-K-R-T or K-F-R-E-L-I-I-E-F-S-K-M-A-R-D. The peptide having the sequence (SEQ ID NO:6) K-F-R-E-L-I-I-E-F-S-K-M-A-R-D is particularly preferred as a stimulating effector peptide.

Still other methods of the invention are for inhibiting the protein tyrosine kinase activity of human epidermal growth factor receptor by reacting an inhibitory effector peptide of the invention with the growth factor, and for stimulating the tyrosine kinase activity of human epidermal growth factor receptor by reacting a stimulatory effector peptide of the invention with the growth factor.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and the accompanying drawings where:

Figure 1 shows a comparison of portions of the amino acid sequence of four (4) related receptors, the insulin receptor, INS-R, the platelet-derived growth factor receptor, PDGF-R, the EGF receptor, HER-1 and HER-2, a receptor with sequence nearly identical to EGF receptor in the cytoplasmic kinase domain (boxes indicate regions of sequence homology).

Figure 2 is a table listing the 90 peptides and their derivatives synthesized of which 78 were tested as described in Examples 1 and 2, infra.

Figure 3 shows the peptides synthesized to cover various segments of the amino acid sequence of EGF receptor from residue 646 to residue 1000, as described in Example 1, infra (peptides having substantial α-helical or β-pleated sheet forming structure are boxed; those having substantial α-helical structure are boxed and shaded. Some of the peptides extend past the boundaries of the sequence at the end of each line and are to be read contiguously from line to line.)

Figure 4 is a bar graph depicting the results of inhibition tests of certain peptides listed in Figure 2, as described in Examples 1 and 2, infra.

Figure 5 is a table summarizing the properties of inhibitors of EGF receptor kinase activity with greater than 34% inhibition occurring at about 1 mM concentrations of peptide, as described in Example 1, infra.

Figure 6 is a table summarizing the properties of additional peptide inhibitors for which maximal inhibition was about 50% or less regardless of peptide concentration, as described in Example 1, infra.

Figure 7 are graphs depicting the inhibitory effects of peptide numbers 32, 41A, 43 and 21, as described in Example 1, infra.

Figure 8 is a graph of the inhibitory effects of peptides 14 and 44 classified as partial inhibitors, as described in Example 1, infra.

Figure 9A, B and C are double reciprocal plots of

the rate of substrate angiotensin II phosphorylation by EGF receptor incubated with different concentrations of substrate and in the absence or presence of inhibitor peptides, as described in Example 1, infra.

Figure 10 is a graph showing the effects of peptide numbers 42 and 26 on substrate phosphorylation by EGF receptor in the presence or absence of EGF, as described in Example 2, infra.

Figure 11 is a diagrammatic representation of the possible three dimensional structure of peptide numbers 26 and 42 represented on a peptide wheel, as described in Example 2, infra.

DETAILED DESCRIPTION OF THE INVENTION

Surprisingly, we have found that incubation of an allosteric protein with certain peptides substantially identical to selected regions of the primary amino acid sequence of the protein, without prior confirmation of specific structural or functional significance of these regions, alters a functional activity of the allosteric protein.

The present invention provides a method for rapidly obtaining peptides capable of altering a functional activity of an allosteric protein, as well as the effector peptides produced by this method. These peptides capable of alteration of an activity of an allosteric protein are referred to as "effector peptides," and sequences in the allosteric protein from which these peptides are derived are referred to as "target sequences." A "functional activity" of an allosteric protein is defined herein as the rate of enzymatic activity if the protein is an enzyme and as the binding affinity of a ligand, e.g. ion molecule or activator, for the protein, expressed as a binding

constant, if the protein is not an enzyme. The alteration of the activity can be either positive, i.e. activating, as evidenced by an increase in enzymatic activity or ion transport, or an increase in binding affinity of the protein for a particular ligand, or it can be negative, i.e. inhibiting, such as a decrease in enzymatic activity or ion transport, or in binding affinity for a ligand.

In addition, the invention provides a method for employing the effector peptides of the invention for inhibiting or activating allosteric proteins.

The Allosteric Protein

The method of the present invention is believed to be applicable to any allosteric protein -- that is, any protein that is sufficiently flexible that an interaction between one molecule and the protein effects, in some manner, a change within the three-dimensional structure of the protein altering activity at other sites.

Typically, the method is applicable to proteins comprising at least one binding site for an allosteric modifier, such as ligands, including sites on the same protein, additional proteins, ions or DNA. The protein can, and most commonly does, have more than one subunit. The binding site for the allosteric modifier need not be located on the same subunit for which a portion of the amino acid sequence is determined and to which the peptide corresponds. Alternatively, the protein can be a monomer at some stage and undergo oligomerization in response to some signal or stimulus. An example is EGF receptor, in which activation by EGF may proceed through oligomerization (Yarden and Schlessinger, Biochem. 26:1434-1442 and 1443-1451 (1987); Fox et al., J. Cell. Biochem. Suppl. 11A, page 32, abstract A, 145A (1987) and Fay and Fox, J. Cell. Biochem. Suppl. 11A, page 32, abstract A, 145B (1987)).

In particular, in allosteric proteins to which the present invention is applicable, there are at least two sites at which other molecules may interact. One of these sites binds an allosteric modifier, and the second site is regulated by binding of the first site to the allosteric modifier and binds to another molecule which is coupled to the expression of activity of the allosteric protein. The interaction of the allosteric modifier and the protein alters the interaction between the protein and the second molecule whether or not the second interaction necessarily occurs subsequent to the first interaction. The interaction of the sites of the allosteric protein with molecules that bind does not have to be sequential.

The allosteric protein may have both intracellular and extracellular domains, i.e., in the cytoplasm of the cell, or on the exterior of the cell surface or within the cell membrane, and the regions of intramolecular interaction may occur in either the intracellular or extracellular domain or in both domains.

The method of the present invention is applicable to allosteric proteins with a wide range of functions, including, but not limited to: enzymes, transport proteins, nucleic acid binding proteins, and receptors. Among the allosteric proteins to which this method is believed applicable are receptors having enzymatic activity including, but not limited to, epidermal growth factor (EGF) receptor including the human EGF receptor, HER-1, insulin receptor (INS-R), platelet-derived growth factor (PDGF) receptor, tumor necrosis factor (TNF) receptor, erythropoietin receptor, receptors for lymphokines such as interleukin-1 (IL-1), and other protein kinases coded for by oncogenes. In particular, the method of the present invention is applicable to the protein tyrosine kinase activity of EGF receptor as set forth in Examples 1 and 2.

I. GENERAL DESCRIPTION OF THE SCREENING METHOD

The screening method of the invention includes A) selection of target sequences in the primary amino acid sequence of an allosteric protein; B) production by synthesis of peptides corresponding to portions of the target sequence; and C) testing of the synthesized peptides for ability to alter a biological activity of the allosteric protein by means other than direct competition for substrate binding.

A. Selection of Target Sequences in the Allosteric Protein For Generation of Effector Peptides

1. Determination of Primary Amino Acid Sequence of Target Region

The effector peptides are substantially identical to a selected region of the amino acid sequence encoding the allosteric protein. This region of the allosteric protein is termed the "target sequence."

In many cases, the amino acid sequence of the allosteric protein is known. If it is not already known, the amino acid sequence of the protein or a selected region of the protein is determined. The determination of amino acid sequence is performed by methods well-known in the art. Classically, amino acid sequences of proteins are determined by methods employing sequential degradation, such as the Edman degradation employing phenyl isothiocyanate. These methods can be automated and performed in a commercially available device known as a "protein sequenator," as described in Niall, "Automated Edman Degradation: The Protein Sequenator," Methods Enzymol. 27, 942-1010 (1973), incorporated herein by this reference.

These classical methods have been recently supplanted by genetic engineering methods that allow cloning of genes coding for particular proteins and then direct determination of the DNA sequence for these genes. These methods are described, for example, in Perbal, "A Practical Guide to Molecular Cloning" (2d ed., John Wiley, New York, 1988), incorporated herein by this reference. The DNA sequence can then be converted directly into the amino acid sequence of the protein by applying the genetic code to groups of three bases. This method is in many cases simpler and more rapid than actual determination of the amino acid sequence because of recent advances in the sequencing of DNA.

An important advantage of the method of the present invention is that the entire amino acid sequence need not be known; knowledge of a region of the sequence will suffice. This portion can be as short as about three amino acids. Moreover, it is not necessary to know the three-dimensional (tertiary or quaternary) structure of the allosteric protein, or to verify the functional or structural relationship of the region to the rest of the protein molecule to apply the method of the invention for producing effector peptides. Obtaining such information most commonly requires use of very expensive and time-consuming X-ray diffraction methods or extensive examination of properties of site-specific mutations in the target sequence.

2. Selection of Target Sequence

Once the amino acid sequence of a region of the primary amino acid sequence of the allosteric protein is determined or known, selection of a target sequence for generating effector peptides is performed. In large proteins, for which some structural information is available, it may be worthwhile to begin the screening in

regions of the protein known to be altered structurally in the generation of allosteric effects. However, this is not required.

The target sequence may be located in an extracellular or intracellular domain of the protein. The target sequence may be contained within, or itself contain one or more, sequence(s) of ordered structure, such as an α-helix or a β-pleated sheet. However, the presence of such ordered structure is not a requirement, and peptides corresponding to regions in the allosteric protein where ordered structure is not predicted are also effective. Additional potential properties of the region of amino acid sequence selected for synthesis of peptides include regions bearing net positive charges, e.g. basic amino acid residues, hydrophobic regions and those capable of functioning as binding sites for ATP, substrates, or other interacting ligands or protein molecules. The selected region may also include substrate binding regions of the allosteric protein, however, this is not required. These regions having putative structural significance are most readily predicted by computational analysis. Regions of functional significance may be identified by sequence homology to known functional domains, e.g. ATP or substrate binding, of related proteins.

The choice of target sequences for peptide synthesis may thus be based on discrete secondary structural features that may be maintained in allosteric structure rearrangements occurring when the protein is activated or that might participate in allosteric conformational interactions within the protein. A recent analysis of protein subunit and domain interactions (Argos, Protein Engineering 2:101-113 (1988)) has shown that interface interactions do not usually involve long stretches of residues with a given secondary structure, and that less than about 70% of the total interface surface is

contributed by single residues in distinct structural units. Consequently, the choice of peptides may be based on a Chou-Fasman secondary structural analysis (Chou & Fasman, Adv. Enzymol. 47:45-148 (1978); Kyte and Doolittle, J. Mol. Biol. 157:105 (1982)), or on a consensus of secondary structural analyses (e.g. using commercially available computer programs such as Protoplot™, Intelligenetics, Inc., Mountainview, CA).

Although predictions of functional or structural significance of certain continuous amino acid sequences may provide guidelines for targeting regions of the amino acid sequence of the allosteric protein for screening, the method of the invention does not require confirmation of the predicted functional or structural significance of the selected regions prior to carrying out the generation of peptides and subsequent screening of the peptides for effects on activity of the protein. The method of the invention thus permits generation of a plurality of peptides having overlapping sequence and accounting for the entire sequence of the target region in essentially two steps, and screening of these peptides for effects on activity of the protein, for rapid identification of those peptides capable of altering function. This method permits the efficient production of new effector peptides from regions of a protein not previously identified as possessing a specific function or structural relationship without requiring the complete amino acid sequence or structural analysis of the protein. Such peptides may be used as lead compounds in the design of new therapeutic agents to alter activity of allosteric proteins involved in human diseases or dysfunctions.

B. Production of Effector Peptides

The effector peptides suitable for altering the biological activity of the allosteric protein are

relatively small and substantially identical to a selected region of the allosteric protein whose biological activity is to be altered.

1. Sequences of the Effector Peptides

The sequences of the effector peptides are derived from the selected target region of the allosteric protein suspected of having structural and/or functional activity relative to a functional activity of the protein as described above.

2. Size of the Peptides Used for Screening

In order to rapidly generate a number of peptides for screening, and to ensure that the peptides taken together encompass the entire target sequence, and that individual peptides are likely to encompass a continuous sequence of amino acids encoding a site of functional or structural significance, i.e. an alpha helix or hydrophobic region, a first substep is used in which a first set of peptides of from about 11 to about 20 amino acids in length are synthesized, followed by a second substep of synthesis to produce a second set of peptides of from about 11 to about 20 amino acids in length. Each peptide represents a portion of the target sequence of the allosteric protein. The second set of peptides represent peptides that overlap the sequence gaps of the first set of peptides such that points of discontinuity in amino acid sequence between peptides resulting from the first step of synthesis are included in the second set of peptides and are flanked by at least 5 amino acids on either side.

The peptides synthesized in these two substeps are thus partially overlapping, typically for about 5 to 7 amino acids, to detect effector sequences that would otherwise be split between two adjacent peptides. Additionally, extended regions of continuous amino acid

sequence that are predicted to form α-helices or β-pleated sheets are preferably retained within one peptide to the extent possible.

This two step synthesis procedure permits the rapid generation of a plurality of peptides together having sequences that encompass every possible peptide of about 11 amino acids in length within the entire target region selected in the allosteric protein in order to assess the activity of all possible peptides six to seven amino acids in length. All of these peptides that are generated by performing both steps of synthesis are tested for effects on activity of the allosteric protein.

3. Degree of Identity of the Sequence of the Peptides With the Sequence of the Protein

Typically, the sequences of the peptides are identical or substantially identical to portions of the sequence of the corresponding region of the protein, i.e., the target sequence. However, this is not a requirement, and some differences between the sequence of the protein and the corresponding sequence of each peptide can occur as long as the peptide is substantially structurally analogous to the modifier sequence of the protein -- i.e., the peptide assumes a three-dimensional conformation virtually identical to the predicted conformation of the corresponding segment of the protein, despite the occurrence of changes in amino acid sequence between the protein and the peptide. These changes can include, but are not limited to, the changes that would result from single basepair changes (i.e., transitions and transversions) in the DNA sequence coding for the modifier sequence.

The required degree of substantial structural analogy preferably exists over the entire length of the peptide; however, if the target sequence includes a dominant structural feature that does not encompass the entire length of the target sequence, duplication of that dominant structural feature can suffice to generate substantial structural analogy, even though the other portions of the effector peptide are somewhat divergent in sequence. The existence of substantial structural analogy can often be predicted by consideration of size, charge, and relative hydrophobicity of the amino acids involved. Certain changes in amino acid residues, known in the art as "conservative amino acid substitutions," result in substantial structural analogy in most cases. Such substitutions include, but are not necessarily limited to: glutamic acid (Glu or E) for aspartic acid (Asp or D) and vice versa; glutamine (Gln or Q) for asparagine (Asn or N) and vice versa; serine (Ser or S) for threonine (Thr or T) and vice versa; and any of isoleucine (Ile or I), valine (Val or V), and leucine (Leu or L) for any other of these amino acids.

4. Synthesis of the Peptides for Testing

The peptides are synthesized by methods well-known in the art. The universally adopted method of choice for synthesis is the solid-phase synthesis protocol developed by Merrifield, as described in Merrifield, J. Am. Chem. Soc. 85: 2149-2154 (1963), incorporated herein by this reference. Variations on this method have enhanced the versatility of the solid-phase synthesis technique. These variations allow the simultaneous synthesis of several peptides of varying sequence and are therefore particularly useful.

The first of these variations of the Merrifield method was described by Geysen and coworkers in 1984 in Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1984), incorporated herein by this reference. This method uses polyethylene rods to which acrylic acid is photocoupled to provide a "handle" on which a given peptide is built. Since, except for the coupling step, all other steps in the solid-phase peptide synthesis are independent of peptide sequence, several such rods can be used concurrently, each rod for a single peptide.

The second such variation of the Merrifield method was described by Houghten in 1985 in Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985), incorporated herein by this reference. This method is described in detail in Example 1, below, as applied to peptides corresponding to sequences in the EGF receptor.

Other methods include those described by Atherton and Sheppard, in Solid Phase Peptide Synthesis, a Practical Approach, IRL Press, Oxford University Press, Oxford, New York, Tokyo (1989); and Stewart and Young, in Solid Phase Peptide Synthesis, 2nd Edition, Pierce Chemical Co., Rockford, IL (1984), both incorporated by reference herein.

The carboxyl-terminal and amino-terminal residues of the synthesized peptides are preferably blocked to avoid the presence of undesirable charges on these residues that might alter the binding of the peptides to the allosteric protein.

Also expected to perform satisfactorily are chemically altered derivatives or analogs of peptides corresponding to target sequences. These derivatives include, but are not limited to, derivatized peptides in which charged residues such as arginine are modified to balance positive charge so that the peptides can penetrate

the cell membrane more efficiently and peptides in which the amino-terminus, the carboxyl-terminus, or both are blocked to prevent the introduction of unwanted charges. Other derivatives of modifier sequence peptides can include peptides in which carboxyl, hydroxyl, or sulfhydryl functions are protected or blocked. As used herein, the term "peptides" embraces generically both underivatized peptides and derivatives or analogs of peptides.

C. Testing of the Synthesized Effector Peptides for Alteration of Activity of the Allosteric Protein

Because the small peptides used in the screening method are generally stable after synthesis, it is not necessary to perform the selection and synthesis steps with the testing steps at the same time or in the same location. The peptides can be stored after synthesis, as a lyophilized powder or in a refrigerated or frozen solution for later reaction with the allosteric protein.

For reaction with the allosteric protein to determine activity, each of the synthesized peptides is added to the allosteric protein in a separate reaction mixture and the effect of the peptide on the activity of the protein is determined by assay after incubation. The reaction is carried out as described below. The assay for activity can take the form of an enzymatic assay, such as an assay of kinase or phosphatase activity, if the protein is an enzyme, or a ligand-binding assay, such as an assay of the binding of a hormone to a receptor, if the protein has such activity. Typically, peptides that alter the activity of the allosteric protein by at least 50%, at a peptide concentration of 1 mM, are chosen for further study in order to determine the most effective peptides. This 50% criterion can be varied for particular allosteric proteins; in some circumstances, an alteration of 25% of the activity of the allosteric protein at 1 mM peptide can

be significant and useful.

Once the peptides showing effector activity have been obtained, their effect can be quantitated by straightforward dose-response assays, in which the concentration of the peptide is varied and the concentration of peptide giving half-maximal response is determined. This concentration, designated EC_{50}, gives an estimate of the association constant of the peptide-allosteric protein complex.

1. Reaction of the Peptide with the Allosteric Protein

To effect the alteration of a biological activity of the allosteric protein, the protein is reacted with a stoichiometric excess of the peptide in an aqueous medium. The temperature of the incubation is between about 0°C to the minimum temperature at which the allosteric protein is denatured or ceases to display its allosteric behavior, typically between about 0°C to about 40°C, and more typically between about 20°C and about 37°C. The pH of the medium is from about 5 to about 10, typically from about 6 to about 8.5, preferably from about 7 to about 8, and more preferably from about 7.2 to about 7.8. This pH can be maintained by a suitable buffer that does not interact with the protein or the peptide, such as Tris or HEPES; other buffers can also be used. The ionic strength of the solution is typically less than about 0.1. Divalent cations such as Mg^{2+}, stabilizers such as bovine serum albumin (BSA), other salts, antioxidants, or other components can be added to the solution as needed to enhance the stability of the protein or for assay of its activity. The molar concentration of peptide used in the reaction is typically at least 0.1 mM, but generally no greater than 1 mM; more typically, it is at least 0.5 mM. The time of reaction is generally noncritical, and

typically ranges from 1 minute to 1 hour.

Reactions that are carried out for from between about 1 minute to about 1 hour at a concentration of peptide of no greater than about 1 mM at a temperature of from about 0°C to the minimum temperature at which the allosteric protein is denatured or ceases to display its allosteric behavior and a pH of from about 5 to about 10 are described herein as reactions under standard conditions.

III. METHOD FOR USING THE EFFECTOR PEPTIDES TO ALTER ACTIVITY

The effector peptides obtained from performance of the above-described screening method of the invention, and shown by testing to affect a functional activity of the allosteric protein are used to react with the allosteric protein to alter the expression of its activity. The conditions of reaction will be generally as described above for testing of the synthesized effector proteins. Effector peptides shown to possess inhibiting activity are used to inhibit the allosteric protein, and peptides possessing stimulatory activity are used to activate the protein.

An additional application of the method of the invention for altering activity of an allosteric protein is to alter the relative activity of an allosteric protein toward different substrates. For example, EGF receptor can catalyze both autophosphorylation and phosphorylation of tyrosine residues on exogenous peptides or proteins, and the relative inhibition of autophosphorylation and phosphorylation of exogenous peptides can vary as between different effector peptides (Example 1).

Therefore, an extension of the screening method of the invention is to determine the modification by

effector peptides of activity of the allosteric protein toward two or more different substrates, such as different target substrates of a protein kinase, and then select effector peptides that have a differential effect as between the two substrates. This can be done by defining the activity of the allosteric protein as the activity toward the first substrate divided by the activity toward the second substrate, and then selecting effector peptides that maximize the activity thus defined. It may even be possible to find effector peptides that cause inhibition toward one substrate and activation toward another substrate.

In addition, many known allosteric proteins are members of protein families that are related in structure and function. For example, about 50 protein tyrosine kinases are known, and these proteins share substantial sequence homology. As shown in Figure 1, the insulin receptor (INS-R), the platelet derived growth factor receptor (PDGF-R), and the human EGF receptor HER-1, and HER-2, are highly homologous in primary amino acid sequence in the kinase domain. This homology suggests that effector peptides can be developed using the methods of the invention that affect the activity of more than one of the allosteric proteins in a family by focusing on regions of substantial sequence homology between the family members. In some cases, these regions of substantial homology can take the form of a "consensus sequence" that is identical or substantially identical for all members of the family; however, the presence of a consensus sequence is not a requirement for the application of the alteration method to a family of allosteric proteins. Effector peptides that can affect the activity of more than one member of a family of allosteric proteins are produced by identifying the region (or regions) of substantial homology of each allosteric protein belonging to the family of allosteric proteins; synthesizing a plurality of peptides, each

peptide substantially identical to a portion of the region of substantial homology between each allosteric protein; and reacting each peptide synthesized with each of the allosteric proteins under the standard conditions for the modification assay to determine the activity of each of the allosteric proteins subsequent to reaction with each peptide to obtain the peptides that alter the activity of each of the allosteric proteins in the family of allosteric proteins by at least a predetermined fraction, typically 25 to 50% at a concentration of peptide of 1 mM.

In a variation of this method, effector peptides may be synthesized from a target sequence in an allosteric protein that is a member of a family of proteins having sequence homology, where the target sequence does not possess homology to sequences in other members of the family. Such non-homologous effector peptides can be predicted to specifically alter the activity of one or a few members of the family, but not others.

IV. APPLICATION TO EPIDERMAL GROWTH FACTOR RECEPTOR (EGF)

Application of the methods of the invention for screening and altering protein activity is exemplified by alteration of the protein tyrosine kinase activity of epidermal growth factor (EGF) receptor. Both inhibition and activation of this enzymatic activity can be produced by incubation with effector peptides having different amino acid sequences. Full details of the application of this method to EGF receptor are given in Examples 1 (inhibition of EGF receptor protein tyrosine kinase activity) and 2 (activation of EGF receptor protein tyrosine kinase activity), infra.

A. Peptides Capable of Inhibiting Protein Tyrosine Kinase Activity of EGF Receptor (Example 1)

The following peptides are capable of inhibiting the protein tyrosine kinase activity of human EGF receptor by at least 34% when incubated with the receptor at about 1 mM concentration of the peptide and for which inhibition has been confirmed in dose-response assays (amino acid residues of EGF receptor and peptide numbers are indicated in parentheses) (SEQ ID NO:6) V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W (693-707, 49); T-V-Q-L-I-T-Q-L-M-P (761-770, 14); W-C-V-Q-I-A-K-G-M-N-Y-L (793-804, 5); G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L (800-814, 6); V-K-I-T-D-F-G-L-A-K-L-L-G (827-839, 10); M-A-L-E-S-I-L-H-R-I-Y-T (857-868, 44); Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M (870-884, 50); P-A-S-E-I-S-S-I-L-E-K (895-905, 21); P-I-C-T-I-D-V-Y-M-I-M-V-K-C (913-926, 48); W-M-I-D-A-D-S-R-P-K-F (927-937, 39); K-F-R-E-L-I-I-E-F-S-K-M-A-R-D (936-950, 26); F-Y-R-A-L-M-D-E-E-D-M-D (973-985, 27); D-D-V-V-D-A-D-E-Y-L-I-P (984-995, 17); D-E-Y-L-I-P (990-995, 41A); N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K (676-690, 18); T-E-F-K-K-I-K-V-L-G-S-G-A (686-698, 45); A-K-L-L-G-A-E-E-K-E-Y-H-A-E-G (835-849, 11); K-V-K-I-P-V-A-I (713-720, 32); I-T-Q-L-M-P-F-G-C-L-L-D (765-776, 23); C-L-L-D-Y-V-R-E (773-780, 28); V-Q-I-A-K-G-M-N-Y-L (795-804, 5A); A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T (815-830, 43); I-M-V-K-C-W-M-I-D-A-D (922-932, 25); P-L-T-P-S-G-E-A-P (667-675, 13); Y-L-V-I-Q-G-D (954-960, 30A); and D-E-Y-L-I-P-Q-Q-G-F-F (990-1000, 41) (see Example 1, and Figure 2, infra). Derivatives of these peptides, are also expected to display comparable activity.

Of these peptides, the following are preferred, being capable of inhibiting the protein tyrosine kinase activity by at least 50% at about 1 mM concentration of the peptide (peptide numbers are indicated in parentheses) (SEQ ID NO:6): T-V-Q-L-I-T-Q-L-M-P (14); K-V-K-I-P-V-A-I (32); I-T-Q-L-M-P-F-G-C-L-L-D (23); C-L-L-D-Y-V-R-E (28); W-C-V-Q-I-A-K-G-M-N-Y-L (5); V-Q-I-A-K-G-M-N-Y-L (5A); G-M-N-Y-L-

E-D-R-R-L-V-H-R-D-L (6); A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T (43); P-I-C-T-I-D-V-Y-M-I-M-V-K-C (48); I-M-V-K-C-W-M-I-D-A-D (25); Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M (50); V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W (49); K-F-R-E-L-I-I-E-F-S-K-M-A-R-D (26); D-D-V-V-D-A-D-E-Y-L-I-P (17); N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K (18); T-E-F-K-K-I-K-V-L-G-S-G-A (45); Y-L-V-I-Q-G-D (30A); and D-E-Y-L-I-P-Q-Q-G-F-F (41).

The following peptides are capable of inhibiting the protein tyrosine kinase activity by at least 75% at about 1 mM concentration of the peptide (peptide numbers are indicated in parentheses) (SEQ ID NO:6): K-V-K-I-P-V-A-I (32); I-T-Q-L-M-P-F-G-C-L-L-D (23); W-C-V-Q-I-A-K-G-M-N-Y-L (5); V-Q-I-A-K-G-M-N-Y-L (5A); G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L (6); A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T (43); Y-L-V-I-Q-G-D (30A); and I-M-V-K-C-W-M-I-D-A-D (25).

The most highly preferred inhibitors (SEQ ID NO:6): A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T (43), Y-L-V-I-Q-G-D (30A) and V-Q-I-A-K-G-M-N-Y-L (5A), corresponding to amino acid residues 815-830, 954-960 and 795-804, respectively, of EGF receptor, when present at about 1 mM concentration of peptide, produce a greater than 85% inhibition of the protein tyrosine kinase activity of EGF receptor.

B. Peptides Capable of Stimulating the Protein Tyrosine Kinase Activity of EGF Receptors

Several effector peptides are capable of activating the protein tyrosine kinase activity of EGF receptor: a peptide with a sequence of (SEQ ID NO:6) R-R-H-I-V-R-K-R-T, corresponding to residues 646-654 of the EGF receptor (peptide number 42), and a peptide with a sequence of (SEQ ID NO:6) K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, corresponding to residues 936-950 of the EGF receptor (peptide number 26).

In addition to effector peptides reactive with the EGF receptor, effector peptides reactive with other allosteric enzymes such as ATCase may be produced. For this enzyme, allosteric transitions occur between a tense (T) form of enzyme with low affinity substrate binding and low specific activity which is in equilibrium with a relaxed (R) form which has high affinity substrate binding and high specific activity. Conversion from the T to R state is induced by either substrate. Several regions of the molecule are engaged in homotypic or heterotypic transitions from T to R state induced by substrate and are good candidates for target sequences for peptide inhibitors or stimulators. These sequences include, on the Asp domain, the sequence Lys_{164} to Ser_{171} in which these residues and Tyr_{165} and Arg_{167} are involved in points of homotypic or heterotypic contact engaged in the allosteric transition, the sequence Arg_{229} to Tyr_{240} in which these residues and Glu_{233}, Arg_{234} and Glu_{239} are engaged in contacts influenced by the allosteric transition, a sequence involving Ser_{171} and a sequence involving Asp_{271}-Glu_{272}. These sequences on the CP domain include one in which Gln_{133} and His_{134} are represented as well as two others containing Glu_{50} and Arg_{105}.

V. THEORY OF OPERATION

Although not wishing to be bound by this theory, at least some of these peptides affecting the protein tyrosine kinase activity of EGF receptor may operate by altering the intermolecular interaction between individual monomers of the receptor that result in the oligomerization of EGF receptor. This interaction may occur at small, discrete recognition points on the EGF receptor monomer. Some of these recognition points are likely to occur within the cytoplasmic region of EGF receptor, but others may be extracellular. However, it seems unlikely that all the peptides that inhibit protein tyrosine kinase activity operate by inhibiting receptor oligomerization. Other

mechanisms, such as intramolecular structural alterations or inhibition of intramolecular flexibility required for allosteric signal transmission, may well account for the activity of some of the inhibitory peptides and of the activating peptides.

VI. APPLICATION OF METHODS OF THE INVENTION TO DEVELOPMENT OF NEW REAGENTS FOR THERAPY

The rapid screening method of the invention shows promise as a general screening method for selecting effector peptides that function as new therapeutic drugs aimed at specific allosteric proteins, or families of proteins. Moreover, once effector peptides of particular sequence are identified, selective inhibitor and activator reagents may be developed for allosteric proteins using computational chemistry. In addition, the portions of the target sequences in an allosteric protein that correspond to effector peptides identified by the method, may be investigated for heretofore unidentified structural or functional significance.

The following examples are included for illustrative purpose only and are not intended to limit the scope of the invention.

Example 1

Inhibiting Protein Tyrosine Kinase Activity of Human Epidermal Growth Factor Receptor with Synthetic Peptides Derived from the Catalytic Domain

Selection of Target Sequence for Synthesis of Effector Peptides

Reagents

Synthetic human angiotensin II was purchased from Sigma Chemical Co. (St. Louis, MO). t-Butyloxycarbonyl (t-BOC) derivatives of amino acids were obtained either from Peninsula Laboratories (Belmont, CA) or from Fisher Scientific Co. (Pittsburgh, PA). N,N-Diisopropylethylamine and 1,3-diisopropylcarbodiimide were purchased from Aldrich Chemical Co. (Milwaukee, WI) and 4-methylbenzhydrylamine resine from Biosearch (San Rafael, CA). Trifluoroacetic acid was obtained from Pierce (Rockford, IL) and Fisher Scientific Co. Sheets of 74 μm pore size nylon mesh used for construction of "teabags" for simultaneous multiple peptide synthesis were obtained from McMaster and Carr, Los Angeles, CA.

Selection of Peptide Sequences

The amino acid sequence of human epidermal growth factor receptor (EGF receptor) from amino acid residue 646 to residue 1000 is shown in Figure 1. This region is generally considered to encompass the protein tyrosine kinase and substrate binding domains (Yarden & Ullrich, Ann. Rev. Biochem. 57:443-478 (1988)). The (SEQ ID NO:6) G-S-G-A-F-G sequence (residues 695-700), the lysine residue at position 721, and the (SEQ ID NO:6) D-F-G sequence (residues 831-833) are all known to participate in ATP binding (Russ et al., J. Biol. Chem. 260:5205-5208 (1985); Sternberg & Taylor, FEBS Letters 175:387-392 (1984); Vogel et al. Eur. J. Biochem. 154:529-532 (1986)). The substrate binding domain is thought to be contained in the remaining sequence spanning residues 834 to approximately 1000 (Yarden & Ullrich, Ann. Rev. Biochem., supra). Previous work by Fox et al., J. Cell. Biochem. Suppl. 11A, page 32, abstract A, 145A (1987) and Fay and Fox, J. Cell. Biochem. Suppl. 11A, page 32, abstract A, 145B (1987), had indicated the obligatory roles of both receptor oligomerization and cooperative interactions mediated by high ATP concentrations on activation of the kinase activity.

In choosing target sequences for peptide synthesis, secondary structural features which might play an important role in receptor-receptor recognition when the EGF receptor oligomerizes on activation by EGF or that might participate in allosteric conformational interactions within the receptor, were maintained where possible. A recent analysis of protein subunit and domain interactions (Argos, Protein Engineering 2:101-113 (1988)) has shown that interface interactions do not usually involve long stretches of residues with a given secondary structure, and that less than about 70% of the total interface surface is contributed by single residues in distinct structural units. Consequently, the choice of peptides was based on secondary structural analyses (Chou & Fasman, Adv. Enzymol. 47:45-148 (1978); Kyte and Doolittle, J. Mol. Biol. 157:105 (1982)). All stretches of 10 amino acid residues or more that occurred either as an α-helix or a β-pleated sheet were retained intact. In regions of intermediate or more random structure, sequences of 8 to 16 residues were chosen. Once these selections were made, a third set of sequences that overlapped two neighboring sequences was also generated. With two exceptions, the overlap included at least three residues on neighboring sequences. This resulted in initial synthesis of a total of 56 peptides that spanned the entire region from amino acid residue 646 to residue 1015. These peptides are shown in the table of Figure 2 and Figure 4. Figure 2 lists the peptides in order of sequence (Protoplot™ was used to predict structure for the peptides shown in Figure 2). Figure 3 shows the relationship of the peptides with respect to the amino acid sequence of the EGF receptor, showing the overlaps between peptides. Peptides containing regions with substantial α-helical or β-pleated sheet structure are boxed; those with substantial α-helical structure are also shaded.

Synthesis of Peptides

The selected peptides were synthesized employing the simultaneous multiple peptide synthesis (M-SPPS) "teabag" protocol of Houghten and coworkers (Houghten, Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985)), or by traditional solid phase peptide synthesis (SPPS) as described by Merrifield, J. Am. Chem. Soc. 85:2149-2154 (1963), or Stewart and Young, in Solid Phase Peptide Synthesis, 2nd Edition, Pierce Chemical Co., Rockford, IL (1984).

The "teabag" protocol did not lend itself to determination of the degree of coupling at each coupling step. Therefore, the Merrifield synthesis technique was used for synthesis of all peptides for which interesting results were initially obtained by the teabag procedure.

For M-SPPS, 100 mg (0.5 meq/g) amounts of 4-methylbenzhydrylamine (MBHA) resin were sealed in polypropylene 74-μm mesh packets having approximate dimensions of 2 cm x 2 cm. These packets were treated concurrently in a common reaction vessel for the standard deprotection, neutralization, and wash procedures. They were separated at the coupling steps where the contents in each packet was reacted with the appropriate amino acid-activator solution in an individual reaction vessel; coupling was effected with 1,3-diisopropylcarbodiimide. Although individual couplings and deprotections were not monitored, additional dummy packets were processed. At appropriate points during synthesis, a dummy packet was removed, the resin collected and assayed for completeness of either deprotection or coupling by the standard Kaiser ninhydrin test (Stewart and Young, supra).

After the addition of the specified amino acid residues, the protected peptide-resins in the packets were collectively deprotected at the amino-terminus and then acetylated in a solution of N,N-dimethylformamide

containing 10% acetic anhydride and 10% N,N-diisopropylethylamine in dichloromethane (DCM) at room temperature for 1-2 hours.

For SPPS, 1.0 (0.4 - 0.79 meq/g) amounts of 4-methylbenzhydrylamine (MBHA) resin were placed in 45 X 80 mm reaction vessels. Deprotection, neutralization and wash procedures were the same as described for M-SPPS except that indole was included in the deprotection stage at 1 g/1000 ml. Reaction vessels were shaken vigorously rather than with a slow rocking movement. All couplings and deprotections were monitored using the Kaiser ninhydrin test. All syntheses were completed by a final acetylation at the amino-terminus as described for M-SPPS.

Peptides synthesized by M-SPPS were deprotected and cleaved from the resin by anhydrous hydrogen fluoride in the presence of anisole by Multiple Peptide Systems (San Diego, California). SPPS peptides were cleaved from the resin using a Multiple Peptide Systems cleavage apparatus. Scavenger was the same as with M-SPPS except for the use of p-cresol and thiocresol in some cases. Peptides were extracted from resin using either glacial acetic acid or 10% acetic acid and lyophilized.

Purification

Crude peptides were purified by preparative reverse-phase HPLC either on a C-18 (Beckman Ultraprep™, 2.12 x 15 cm) or a C-4 (Vydac, 2.2 x 25 cm) column using an aqueous gradient of 0 to 60% acetonitrile containing 0.1% trifluoroacetic acid. Each peptide was at least 90% pure as analyzed by HPLC; the composition of each peptide was established by amino acid analysis.

Chemical Characterization

Peptides purified by HPLC were analyzed by reverse phase HPLC using conditions similar to those for preparative purification. Peptide samples (2-5 nmol) were analyzed for amino acid composition by the University of California at Los Angeles protein microsequencing laboratory to confirm expected composition.

Purification of EGF

EGF was purified from male mouse submaxillary glands as described in Savage & Cohen, J. Biol. Chem. 247:7609-7611 (1972). Briefly, the isolation procedure involved: (1) chromatography in 0.05 N HCl containing 0.15 M NaCl on Bio-Gel™ P-10 (Bio-Rad Laboratories, Richmond, Calif.); and (2) DEAE-cellulose chromatography.

Tests for Inhibition of Substrate Phosphorylation

The peptides were examined for their inhibitory properties in an EGF-dependent EGF-receptor-catalyzed substrate phosphorylation assay using receptor that had been purified about 500-fold from Triton X-100 extracts of human epidermoid A431 cells by affinity chromatography on Fractogel TSK-immobilized ricin-binding subunit, as described in Ghosh-Dastidar et al., Proc. Natl. Acad. Sci. USA 81:1654-1658 (1984). The reaction system was a modification of the procedure described in Pike et al. Proc. Natl. Acad. Sci. USA 79:1443-1447 (1982). The final 20 μL reaction system contained: 20 nM EGF receptor or 200 nM EGF; 3 mM angiotensin II as phosphorylation substrate; concentrations of peptide ranging from 0 to 1 mM as specified; 50 μM [γ-^{32}P]ATP (1500-3000 cpm/pmoles); 5 mM $MgCl_2$; 10 μg/mL BSA, 0.2% Triton X-100; and 10% glycerol in 10 mM HEPES adjusted to pH 7.4. In each case, control incubations containing all assay components except phosphorylation substrate were included.

Reaction systems containing all components except $MgCl_2$ and ATP in a total volume of 18 μL were incubated at 30°C for 3 minutes to allow ligand-receptor complexes to form. Reactions were initiated by the addition of $MgCl_2$ and [γ-^{32}P]ATP in 2 μL and were incubated at 30°C for 3 minutes. Reactions were terminated by mixing 5 μL aliquots of the reaction mixture with 50 μL of 5% (w/v) trifluoroacetic acid. Phosphorylated receptor protein was sedimented by a 5-minute centrifugation in a microfuge. Thirty μL of each supernatant fraction was adsorbed onto a piece of Whatman P-81 phosphocellulose paper (6.45 cm^2) that was then washed once for 15 minutes in 400 mL of 10% acetic acid, and then thrice for 15 minutes each in 300 mL each of 5% acetic acid and, finally, in acetone prior to being dried in air. Phosphorylated angiotensin bound to the paper was quantified by Cerenkov counting.

As detailed below, some assays were run in the absence of EGF to determine the effect of the tested peptides on EGF-independent, EGF-receptor-catalyzed phosphorylation of angiotensin II. In these assays, peptide concentrations were generally varied from 0.1 to 1 mM, but peptide concentrations as high as 2.5 mM were sometimes used.

Tests for Inhibition of Autophosphorylation

Inhibition of EGF-dependent EGF receptor self-phosphorylation activity by the synthetic peptides was determined with 20 nM EGF receptor and 200 nM EGF in the 20-μL system described for substrate phosphorylation, but with angiotensin II excluded. Reaction systems, complete in 18 μL, except for $MgCl_2$ and ATP, were incubated at 30°C for 3 minutes to allow for ligand-receptor complexes to form and then for an additional 10 minutes at 0°C prior to addition of $MgCl_2$ and [γ-^{32}P]ATP. After 20 seconds at 0°C, reactions were terminated by adding 20 μL of 2-fold

concentrated electrophoresis sample buffer (100 mM Tris, 6% sodium dodecyl sulfate, 40% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol). Phosphorylated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 1.4-mm-thick slab gel of 7.5% polyacrylamide and then located by autoradiography, excised, and quantified by Cerenkov counting.

Results

1. Inhibition of EGF-dependent EGF-receptor-catalyzed Substrate Phosphorylation

The results of the assays for inhibition of EGF-dependent EGF-receptor catalyzed substrate phosphorylation are shown with reference to the linear sequence of the EGF receptor in Figure 4, using 3 mM angiotensin II as phosphorylation substrate and a 1 mM concentration of peptide being tested for its properties as an inhibitor.

Of the 56 peptides initially tested for possible inhibitor activity as shown in Figure 4, and as summarized in Figures 5 and 6, 15 were relatively potent, inhibiting EGF stimulated angiotensin II phosphorylation by 50% or more when present at 1 mM concentration. A peptide (SEQ ID NO:6), Y-L-V-I-Q-G-D (peptide number 30A), was subsequently shown to inhibit phosphorylation by more than 50%. This inhibitory sequence is within the sequence of a larger peptide, peptide number 30, which was among that group of 26 peptides that were weak inhibitors or noninhibitory. Nine other peptides when present at 1 mM concentration inhibited by 34% to 50%, and the remaining 26 peptides were either weakly inhibitory or noninhibitory.

The distribution of the sequences of the 15 relatively potent peptides along the EGF receptor present several interesting features. First, two peptides that

include regions in EGF receptor known to be involved in ATP binding, namely peptides corresponding to EGF sequence positions 693-707 and 827-839 are inhibitors of EGF receptor-catalyzed substrate phosphorylation. Second, of the 15 relatively potent inhibitor peptides, 6 correspond to sequences occurring from residue 834 to 1000, a region thought to contain at least part of the substrate binding domain. Inhibition by at least one of these peptides, corresponding to residues 895-905, is possibly non-competitive with substrate, i.e, with angiotensin II. Three additional peptides were also characterized for type of inhibition, and all were revealed to possess noncompetitive properties as shown in Figure 5. Third, a cluster of four inhibitor peptides which inhibit by at least 40% when present at 1 mM concentration, correspond to sequences spanning a 49-amino-acid stretch from residue 913 to residue 961. This is closely followed by a second stretch of 28 amino acid residues, residues 973 to 1000, that produced a cluster of three additional inhibitor peptides that inhibit by at least 45% when present at 1 mM concentration.

Inhibition by these 15 peptides does not involve inhibition of EGF binding to receptor. Because EGF itself is a stimulator of phosphorylation of angiotensin by the EGF receptor, if the peptides inhibited the binding of EGF to the receptor, that would account for at least some of their inhibitory effects. However, no significant variation in binding of EGF to receptor was observed when 20 nM EGF receptor was incubated with 200 nM [^{125}I]EGF in the presence or absence of 1 mM of the peptides for 1 hour at 22°C. Accordingly, the peptides did not prevent binding of EGF to the receptor.

2. Relative Potency of Inhibitor Peptides

As a refinement of the studies summarized in Figure 4, EGF-dependent EGF-receptor-catalyzed phosphorylation of angiotensin II was examined in the presence of varying concentrations of inhibitor peptides ranging from 0.125 mM to 2.5 mM. Only those peptides that inhibited substrate phosphorylation by 34% or more at a concentration of 1 mM were chosen for this study (Figures 5 and 6). Figure 7 depicts examples of the results of this study. Inhibition is expressed as a function of the effects of the peptide concentration on the specific activity of EGF-receptor catalyzed substrate phosphorylation. The most potent inhibitors of those shown were peptides 32 and 43 causing greater than half-maximal inhibition at concentrations of less than 0.25 mM.

The effects of peptide inhibitors shown in Figure 7 are representative of the effects of the peptide inhibitors shown in Figure 5 plus peptide 30A representing EGF receptor sequence 954-960 (SEQ ID NO:6), Y-L-V-I-Q-G-D. The plots of inhibitory peptide concentration vs. specific activity continue to decrease as a function of increasing inhibitor concentration throughout the entire range of concentrations studies and the shapes of the curves suggest that at very high concentration of peptide inhibitor, complete inhibition will be observed. This behavior is in contrast to qualities of inhibition of peptides presented in Figure 6 and shown in greater detail in Figure 8.

Several peptide inhibitors described in Figure 5 produced unique inhibitory effects. Peptide 49 with sequence (SEQ ID NO:6) P-I-C-T-I-D-V-Y-M-I-M-V-K-C inhibited only that portion of substrate phosphorylation activity induced by EGF. This peptide had no inhibitory effect whatsoever on EGF independent substrate phosphorylation. One other peptide, peptide 27 with sequence (SEQ ID NO:6) F-Y-R-A-L-M-D-E-E-D-M-D, also showed this trend with 2.5-fold greater inhibitory effect

on EGF-dependent substrate phosphorylation than on EGF-independent substrate phosphorylation.

The peptides shown in bold type in Figure 5 are all characterized by the presence of a tyrosine residue raising the possibility that inhibition in response to these peptides might occur through these peptides acting as competing substrates which in their phosphorylated form do not bind to phosphocellulose paper as does phosphorylated angiotensin II. One of these tyrosine containing peptides, peptide 28 with sequence (SEQ ID NO:6) C-L-L-D-Y-V-R-E, and three additional peptides, 32, 43 and 21, which contained no tyrosine residues were tested for competitive vs. noncompetitive inhibition as shown in Figure 9A-C. In Figure 9A, 9B and 9C, intersection of plots on the ordinate (1/v) axis are indicative of inhibition by peptide with substrate for the substrate binding site. Intersection of plots on the abscissa (x axis) are indicative of a competition which is noncompetitive with respect to substrate. Plots A and B show characteristics of noncompetitive inhibition, and plot C shows inhibition which may represent a mixture of competitive inhibition at lower inhibitor concentration, e.g. 0.0625 mM, but noncompetitive at higher concentration, e.g. 0.25 mM. Three of these four peptides, 28, 43 and 21 were, characterized by clear noncompetitive inhibition indicating possible direct inhibitor effects through blocking of allosteric properties of the receptor. Peptide 32 was characterized by a mix of competitive inhibitory quality at lower inhibitor concentration and noncompetitive quality at higher inhibitory concentration. The characteristics of inhibition by other peptides shown in Figure 5 were not determined.

Peptide 26 may be further optimized by substitution of amino acids at position that may affect structure. Experiments by Moe and Kaiser (Biochemistry

24:1971-1976 (1985)) on calcitonin show that activity of calcitonin which contains an amphiphilic α-helix is maintained, and in some cases even enhanced when sequences of portions of an idealized α-helix are substituted for portions of the actual sequence of calcitonin. Moreover, substitution of other amino acids for lysine residues 1 and 11 of peptide 26 and for aspartate and glutamate residues 4, 8 and 15 may provide additional information on those residues that may be pharmacophores required for activity.

3. Peptide Inhibitors which Do Not Strongly Inhibit Substrate Phosphorylation at High Inhibitor Concentration

Seven of the inhibitor peptides were limited in their ability to fully inhibit the phosphorylation of angiotensin substrate by EGF receptor. Figure 7 describes the quality of inhibition representative of these peptides for EGF induced substrate phosphorylation. Maximal inhibition is achieved at inhibitor concentration of 0.25 to 0.50 mM and higher concentrations of inhibitor produced no additional inhibition. This may indicate that these inhibitors induce changes in EGF receptor structure which render the activated form of receptor less functional in substrate phosphorylation, or that these partial inhibitors decrease the concentration of the low activity form of receptor to intermediate levels, or that there are subpopulations of receptor that are insensitive to certain inhibitors. Three additional inhibitors of the seven partial inhibitors also had a quality shared by the two inhibitors for which inhibitory effects are more fully characterized in Figure 8. These five peptide inhibitors selectively inhibited EGF-stimulated substrate phosphorylation, having a much smaller inhibitory effect, and in some cases as represented by peptides 14 and 10, no inhibitory effect on EGF independent phosphorylation of substrate angiotensin II. This quality of selective

inhibition of the EGF-dependent reaction which is the more generally observed quality for partial inhibitors shown in Figure 6 as compared with more fully effective inhibitors shown in Figure 5 indicates selective interference with allosteric activation of receptor kinase activity induced by EGF.

4. Inhibition of EGF-stimulated EGF Receptor Autophosphorylation by Peptide Inhibitors Shown in Figures 5 and 6

The extent to which peptide inhibitors described in Figures 5 and 6 affected the rate of autophosphorylation of purified human EGF receptor induced by EGF was approximately the same as the extent to which the inhibitors blocked phosphorylation of model substrate angiotensin II.

Example 2

Activation of Protein Tyrosine Kinase Activity of Human Epidermal Growth Factor Receptor by Synthetic Peptides

Selection of Peptides for Synthesis

The peptides used were peptide 42, with a sequence of (SEQ ID NO:6) R-R-H-I-V-R-K-R-T corresponding to residues 646-654 of EGF receptor, and peptide 26, with a sequence of (SEQ ID NO:6) K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, corresponding to residues 936-950 of EGF receptor.

Synthesis of Peptides

Peptides were synthesized initially by the simultaneous multiple peptide synthesis protocol of Houghten and coworkers as described in Example 1, supra, and then by classical Merrifield synthesis to obtain data

in the studies described herein.

Purification of EGF

EGF was purified from male mouse submaxillary glands as described by Savage and Cohen, supra (Example 1).

Tests for Activation of Substrate Phosphorylation

The peptides were examined for their activation activity in an EGF-dependent EGF-receptor-catalyzed substrate phosphorylation assay using receptor that had been purified about 500-fold (Example 1, supra). The reaction system contained, in a total volume of 20 μL, 20-30 nM EGF receptor; 1.5 mM angiotensin II as a phosphorylation substrate; 10 μg/mL BSA, 50 μM [γ-^{32}P]ATP (1500-3000 cpm/pmole); 5 mM $MgCl_2$; 200 nM EGF; 0.3 mM peptide 10 or 0.75 mM peptide 43, unless specified otherwise; 0.2% Triton X-100; and 10% glycerol in 10 mM HEPES, pH 7.4. In some experiments, the EGF was omitted or the concentration of angiotensin II, EGF, EGF receptor, or ATP was varied.

Reaction systems, containing all components except $MgCl_2$ and ATP, were first incubated at 30°C for 3 minutes. Reactions were then initiated by the addition of $MgCl_2$ and ATP in a total volume of 2 μL and incubated for 3 minutes at 30°C. Reactions were terminated by mixing 5-μL aliquots of the reaction mixture with 50 μL of 5% (w/v) of trichloroacetic acid. Phosphorylated receptor was sedimented by a 5-minute centrifugation in a microfuge. Aliquots of each supernatant fraction were applied onto Whatman P-81 phosphocellulose paper which was then washed once with 33% acetic acid for 15 minutes, three times with 5% acetic acid for 15 minutes each, and finally with acetone prior to being dried in air.
Phosphorylated substrate bound to the paper was quantified

by Cerenkov counting.

Tests for Effect of Peptides on Binding of EGF by EGF Receptor

EGF receptor and peptide or buffer were incubated for 20-30 minutes at room temperature; then [^{125}I] EGF (to measure total binding) or a mixture of [^{125}I] EGF and 2 μM unlabeled EGF (to measure nonspecific binding) was added. The volume of each sample was adjusted to 20 μL with 10 mM HEPES, pH 7.4, and incubations were for 1 hour at room temperature. Bovine γ-globulin in 10 mM HEPES, pH 7.4 (300 μL) followed by an equal volume of 20% polyethylene glycol in 10 mM HEPES, pH 7.4, was added. Samples were vortexed vigorously and centrifuged in a microfuge. Supernatant fractions were aspirated. Pellets were washed with 500 μL of 20% polyethylene glycol in 10 mM HEPES, pH 7.4, vortexed, and centrifuged; supernatant fractions were aspirated. Radioactivity in the pellets was determined by gamma counting.

Results

Figure 10A and B shows the effects of peptides 42 (10A) and 26 (10B) on substrate phosphorylation by EGF receptor in the presence or absence of EGF. Units are turnover numbers and represent moles of substrate phosphorylated per min per mole of substrate under standard assay conditions.

1. Effect of Peptide 42 on Phosphorylation of Angiotensin II

The effect of peptide 42 at different concentrations (0, 0.25 mM, 0.50 mM, and 0.75 mM) on the phosphorylation of angiotensin II (substrate phosphorylation) in the presence or absence of EGF at

varying angiotensin concentrations is shown in Figure 10A.

Peptide 42 increased substrate phosphorylation by the intrinsic protein tyrosine kinase activity of EGF receptor in the presence or absence of EGF. Maximal stimulation was observed at 0.5 mM with EGF absent, but at 0.25 mM with EGF present. Peptide 42 slightly decreased the stimulatory effect of EGF on the tyrosine kinase activity of EGF receptor at higher concentrations. EGF acted synergistically with peptide 42 to stimulate the tyrosine kinase activity of the EGF receptor.

Peptide 42 was nearly as effective as EGF in stimulating substrate phosphorylation by EGF receptor and more than tripled the activity of EGF receptor activated by EGF.

2. Effect of Peptide 26 on Phosphorylation of Angiotensin II

Peptide 26 is a powerful activator of EGF receptor catalyzed substrate phosphorylation with full response at concentrations less than 0.1 mM (Figure 10B). Peptide 26 is itself a three-fold more effective activator than EGF. Peptide 26 also acts synergistically with EGF, with the activity induced by combinations of the two being greater than the sum of activation induced by each acting separately. Peptide 26 also had inhibitory quality at concentrations much higher than required to achieve full activation.

The predicted structure for peptide 26 may explain its highly effective properties as an activator relative to those of peptide 42. Figure 11 shows the possible three dimensional structure of peptides 26 and 42 represented on a peptide wheel which presents the amino acids in the positions they would be likely to assume if

the peptide has strong alpha helix forming characteristics, which is the case for peptide 26, but not for peptide 42 (see structural predictions in Figure 2). The five highly charged residues of peptide 26 are clustered on one side of the wheel proceeding clockwise from a positively charged group to a group of three negatively charged residues to a positively charged group. The uncharged residues are clustered on the opposing side of predicted structure of the peptide. If this peptide is highly structured as predicted, amino acids in the sequence would enjoy far less rotational mobility around interatomic bonds than would groups of peptide 42, which is far less effective as an activator and for which much higher concentrations are required for maximal activity.

3. Effects of Peptides 42 and 26 on Binding of [^{125}I]EGF to EGF Receptor

The effect of varying concentrations of peptides 42 and 26 on the binding of [^{125}I]EGF to EGF receptor was studied. Neither peptide 42 or 26 increased or decreased the binding of EGF to the EGF receptor.

Because the activators and inhibitors described in examples 1 and 2 are based on sequences within the intracellular (cytoplasmic) domain of human EGF receptor, these activators and inhibitors may be expected to act on sites in that domain. This may prove advantageous because the inhibitors may be even more efficacious for that reason if applied within the context of delivery mechanisms that can be targeted to specific target cells, e.g. tumor cells.

ADVANTAGES OF THE INVENTION

The present invention provides a method for altering the functional activity of any allosteric protein using synthetic peptides without any knowledge of its

detailed three-dimensional structure or even a complete knowledge of its primary amino acid sequence. The peptides used are stable and easy to synthesize in batches containing a number of peptides with different sequences, making it possible to test a large number of candidate peptides simultaneously. The method can be used to either activate or inhibit the allosteric protein whose activity is affected. The invention can be used to affect the activity of a wide variety of allosteric proteins, including: receptors, such as the EGF receptor, the insulin receptor, and the T-cell receptor complex; transport proteins, such as hemoglobin, and oncogene-related protein kinases.

This method promises to permit totally new treatments for such diseases as cancer, AIDS, diabetes, and arthritis, and to speed wound healing and prevent transplant rejection, among other applications. These treatments would operate by utilizing the body's natural defenses and would act in conjunction with current drug treatments.

For example, the abnormally expressed kinases present in many types of cancers could be inhibited, which would slow the growth of the cancer cells and increase their susceptibility to anti-cancer therapies such as radiation or chemotherapy. This could allow smaller doses of anti-cancer drugs or smaller quantities of radiation to be administered.

As another example, AIDS could be treated by activating the T-cell receptor complex to counteract the efficiency of the HIV virus. This treatment could be performed in conjunction with already-available AIDS drugs such as AZT.

Diabetes could be treated by activating the insulin receptor to allow the body to use a scarce supply

of insulin more efficiently. This could reduce the need for injections of insulin and provide more precise control of blood sugar levels, preventing some of the complications associated with diabetes, such as eye damage and circulatory impairment.

The invention can also be applied to target sequences of the extracellular domains of allosteric proteins to identify inhibitors or activators which bind to an extracellular site for applications such as corneal wound healing. In addition, the peptides of the invention can be used as an agent which causes a sheep's wool coat to be shed
as a substitute for sheep shearing.

As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described above, are, therefore, to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Williams, Robert E.

(ii) TITLE OF INVENTION: Novel Peptides and Method for Altering the Activity of Allosteric Proteins

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Arg Arg Leu Leu Gln Glu Arg Glu Lys Val Glu Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Ala Arg Asn Val Leu Val Lys Thr Pro Gln Val Lys Ile Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gln Val Lys Ile Thr
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Lys Ile Thr Asp Phe Gly Lys Ala Lys Lys Lys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Arg Arg Asp Glu Tyr Leu Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 416 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg Arg Arg His Ile Val Arg Lys Arg Thr Leu Arg Arg Leu Leu Gln
1 5 10 15

Glu Arg Glu Leu Val Glu Pro Leu Thr Pro Ser Gly Glu Ala Pro Asn
20 25 30

Gln Ala Leu Leu Arg Ile Leu Lys Glu Thr Glu Phe Lys Lys Ile Lys
35 40 45

Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys Gly Leu Trp Ile
50 55 60

Pro Glu Gly Glu Lys Val Lys Ile Pro Val Ala Ile Lys Glu Leu Arg
65 70 75 80

Glu Ala Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu Asp Glu Ala Tyr
85 90 95

Val Met Ala Ser Val Asp Asn Pro His Val Cys Arg Leu Leu Gly Ile
100 105 110

Cys Leu Thr Ser Thr Val Gln Leu Ile Thr Gln Leu Met Pro Phe Gly
115 120 125

Leu Leu Asp Tyr Val Arg Glu His Lys Asp Asn Ile Gly Ser Gln Tyr
130 135 140

Leu Leu Asn Trp Cys Val Gln Ile Ala Lys Gly Met Asn Tyr Leu Glu
145 150 155 160

Asp Arg Arg Leu Val His Arg Asp Leu Ala Ala Arg Asn Val Leu Val
165 170 175

Lys Thr Pro Gln His Val Lys Ile Thr Asp Phe Gly Leu Ala Lys Leu
180 185 190

Leu Gly Ala Glu Glu Lys Glu Tyr His Ala Glu Gly Gly Lys Val Pro
195 200 205

Ile Lys Trp Met Ala Leu Glu Ser Ile Leu His Arg Ile Tyr Thr His
210 215 220

Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val Trp Glu Leu Met Thr
225 230 235 240

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Phe Gly Ser Lys Pro Tyr Asp Gly Ile Pro Ala Ser Glu Ile Ser Ser
                245                 250                 255

Ile Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro Pro Ile Cys Thr Ile
            260                 265                 270

Asp Val Tyr Met Ile Met Val Lys Cys Trp Met Ile Asp Ala Asp Ser
        275                 280                 285

Arg Pro Lys Phe Arg Glu Leu Ile Ile Glu Phe Ser Lys Met Ala Arg
    290                 295                 300

Asp Pro Gln Arg Tyr Leu Val Ile Gln Gly Asp Glu Arg Met His Leu
305                 310                 315                 320

Pro Ser Pro Thr Asp Ser Asn Phe Tyr Arg Ala Leu Met Asp Glu Glu
                325                 330                 335

Asp Met Asp Asp Val Val Asp Ala Asp Glu Tyr Leu Ile Pro Gln Gly
            340                 345                 350

Phe Phe Ser Ser Pro Ser Thr Ser Arg Thr Pro Leu Leu Ser Ser Leu
        355                 360                 365

Ser Ala Thr Ser Asn Asn Ser Thr Val Val Ala Cys Ile Asp Arg Asn
    370                 375                 380

Gly Leu Gln Ser Cys Pro Ile Lys Glu Asp Ser Phe Leu Gln Arg Tyr
385                 390                 395                 400

Ser Ser Asp Asp Pro Thr Gly Ala Leu Thr Glu Asp Ser Ile Asp Asp
                405                 410                 415
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg Arg Leu Leu Gln
1 5 10 15

Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly Ala Met Pro Asn
20 25 30

Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu Arg Lys Val Lys
35 40 45

Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys Gly Ile Trp Ile
50 55 60

Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile Lys Val Leu Arg
65 70 75 80

Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu Asp Glu Ala Tyr
85 90 95

Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg Leu Leu Gly Ile

100 105 110

Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu Met Pro Tyr Gly
115 120 125

Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg Leu Gly Ser Gln Tyr
130 135 140

Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly Met Ser Tyr Leu Glu
145 150 155 160

Asp Val Arg Leu Val His Arg Asp Leu Ala Ala Arg Asn Val Leu Val
165 170 175

Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe Gly Leu Ala Arg Leu
180 185 190

Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp Gly Gly Lys Val Pro
195 200 205

Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg Arg Arg Phe Thr His
210 215 220

Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val Trp Glu Leu Met Thr
225 230 235 240

Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala Arg Glu Ile Pro Asp
245 250 255

Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro Pro Ile Cys Thr Ile
260 265 270

Asp Val Tyr Met Ile Met Val Lys Cys Trp Met Ile Asp Ser Glu Asp
275 280 285

Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe Ser Arg Met Ala Arg
290 295 300

Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu Asp Leu Gly Pro Ala
305 310 315 320

Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu Leu Glu Asp Asp Asp
325 330 335

Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu Val Pro Gln Gln Gly
340 345 350

Phe Phe

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 402 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg Leu Arg Gln Pro Asp Gly Pro Leu Gly Pro Leu Tyr Ala Ser Ser
1 5 10 15

Asn Pro Glu Tyr Leu Ser Ala Ser Asp Val Phe Pro Cys Ser Val Tyr
20 25 30

Val Pro Asp Glu Trp Glu Val Ser Arg Glu Lys Ile Thr Leu Leu Arg
35 40 45

Glu Leu Gly Gln Gly Ser Phe Gly Met Val Tyr Glu Gly Asn Ala Arg
50 55 60

Asp Ile Ile Lys Gly Glu Ala Glu Thr Arg Val Ala Val Lys Thr Val
65 70 75 80

Asn Glu Ser Ala Ser Leu Arg Glu Arg Ile Glu Phe Leu Asn Glu Ala
85 90 95

Ser Val Met Lys Gly Phe Thr Cys His His Val Val Arg Leu Leu Gly
100 105 110

Val Val Ser Lys Gly Gln Pro Thr Leu Val Val Met Glu Leu Met Ala
115 120 125

His Gly Leu Lys Ser Tyr Leu Arg Ser Leu Arg Pro Glu Ala Glu Asn
130 135 140

Asn Pro Gly Arg Pro Pro Pro Thr Leu Gln Glu Met Ile Gln Met Ala
145 150 155 160

Ala Glu Ile Ala Asp Gly Met Ala Tyr Leu Asn Ala Lys Lys Phe Val
165 170 175

His Arg Asp Leu Ala Ala Arg Asn Cys Met Val Ala His Asp Phe Thr
180 185 190

Val Lys Ile Gly Asp Phe Gly Met Thr Arg Asp Ile Tyr Glu Thr Asp
195 200 205

Tyr Tyr Arg Lys Gly Gly Lys Gly Leu Leu Pro Val Arg Trp Met Ala
210 215 220

Pro Glu Ser Leu Lys Asp Gly Val Phe Thr Thr Ser Ser Asp Met Trp

225 230 235 240

Ser Phe Gly Val Val Leu Trp Glu Ile Thr Ser Leu Ala Glu Gln Pro
245 250 255

Tyr Gln Gly Leu Ser Asn Glu Gln Val Leu Lys Phe Val Met Asp Gly
260 265 270

Gly Tyr Leu Asp Gln Pro Asp Asn Cys Pro Glu Arg Val Thr Asp Leu
275 280 285

Met Arg Met Cys Trp Gln Phe Asn Pro Asn Met Arg Pro Thr Phe Leu
290 295 300

Glu Ile Val Asn Leu Leu Lys Asp Asp Leu His Pro Ser Phe Pro Glu
305 310 315 320

Val Ser Phe Phe His Ser Glu Glu Asn Lys Ala Pro Glu Ser Glu Glu
325 330 335

Leu Glu Met Glu Phe Glu Asn Met Glu Asn Val Pro Leu Asp Arg Ser
340 345 350

Ser His Cys Gln Arg Glu Glu Ala Gly Gly Arg Asp Gly Gly Ser Ser
355 360 365

Leu Gly Phe Lys Arg Ser Tyr Glu Glu His Ile Pro Tyr Thr His Met
370 375 380

Asn Gly Gly Leu Leu Asn Gly Arg Ile Leu Thr Leu Pro Arg Ser Asn
385 390 395 400

Pro Ser

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 407 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gln Lys Lys Pro Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val
1 5 10 15

Ser Ser Asp Gly His Glu Tyr Ile Tyr Val Asp Pro Val Gln Leu Pro
20 25 30

Tyr Asp Ser Thr Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg
35 40 45

Thr Leu Gly Ser Gly Ala Phe Gly Gln Val Val Glu Gly Thr Ala His
50 55 60

Gly Leu Ser His Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu
65 70 75 80

Lys Ser Thr Ala Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu
85 90 95

Lys Ile Met Ser His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu
100 105 110

Gly Ala Cys Thr Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys
115 120 125

Arg Tyr Gly Leu Val Asp Tyr Leu His Arg Asp Leu Val Gly Phe Ser
130 135 140

Tyr Gln Val Ala Asn Gly Met Asp Phe Leu Ala Ser Lys Asn Cys Val
145 150 155 160

His Arg Asp Leu Ala Ala Arg Asn Val Leu Ile Gly Glu Gly Lys Leu
165 170 175

Val Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met Arg Asp Ser
180 185 190

Asn Tyr Ile Ser Lys Gly Ser Thr Tyr Leu Pro Leu Lys Trp Met Ala
195 200 205

Pro Glu Ser Ile Phe Asn Ser Leu Tyr Thr Thr Leu Ser Asp Val Trp
210 215 220

Ser Phe Gly Ile Leu Leu Trp Glu Ile Phe Thr Leu Gly Gly Thr Pro
225 230 235 240

Tyr Pro Glu Leu Pro Met Asn Asp Gln Phe Tyr Asn Ala Ile Lys Arg
245 250 255

Gly Tyr Arg Met Ala Gln Pro Ala His Ala Ser Asp Glu Ile Tyr Glu
260 265 270

Ile Met Gln Lys Cys Trp Glu Glu Lys Phe Glu Thr Arg Pro Pro Phe
275 280 285

Ser Gln Leu Val Leu Leu Leu Glu Arg Leu Leu Gly Glu Gly Tyr Lys
290 295 300

Lys Lys Tyr Gln Gln Val Asp Glu Glu Phe Leu Arg Ser Asp His Pro

305 310 315 320

Ala Ile Leu Arg Ser Gln Ala Arg Phe Pro Gly Ile His Ser Leu Arg
325 330 335

Ser Pro Leu Asp Thr Ser Ser Val Leu Tyr Thr Ala Val Gln Pro Asn
340 345 350

Glu Ser Asp Asn Asp Tyr Ile Ile Pro Leu Pro Asp Pro Lys Pro Asp
355 360 365

Val Ala Asp Glu Gly Leu Pro Glu Gly Ser Pro Ser Leu Ala Ser Ser
370 375 380

Thr Leu Asn Glu Val Asn Thr Ser Ser Thr Ile Ser Cys Asp Ser Pro
385 390 395 400

Leu Glu Leu Gln Glu Glu Pro
405

We claim:

1. A method for producing effector peptides that alter a functional activity of an allosteric protein comprising:

a) determining a target sequence of the primary amino acid sequence of an allosteric protein, said target sequence containing at least one site of intramolecular or intermolecular contact within an allosteric protein, said site involved in an allosteric transition resulting in alteration of the expression of functional activity of the allosteric protein;

b) synthesizing a plurality of screening peptides of from about 10 to about 20 amino acids in length, each peptide substantially identical in sequence to a portion of said target sequence and which in linear array correspond to substantially all of the target sequence of the primary amino acid sequence determined in step a); and

(c) measuring a functional activity of the allosteric protein when reacted with each peptide to identify effector peptides that inhibit or activate a functional activity of the allosteric protein.

2. The method of claim 1 wherein said effector peptides are at from about 3 to about 20 amino acids in length.

3. The method of claim 1 wherein said effector peptides are from about 6 to about 10 acids in length.

4. The method of claim 1 wherein said target sequence contains extended regions of α-helix sheet-forming amino acid sequence.

5. The method of claim 1 wherein said target sequence contains extended regions of β-pleated sheet-forming amino acid

sequence.

6. The method of claim 1 wherein said target sequence contains substantially all of a hydrophobic region of the allosteric protein.

7. The method of claim 1 wherein said target sequence contains substantially all of a random coil region of the allosteric protein.

8. The method of claim 1 wherein said step of measuring a functional activity comprises using an enzymatic assay.

9. The method of claim 1 wherein said step of measuring a functional activity comprises using a cell biological assay.

10. The method of claim 1 wherein said allosteric protein is selected from the group of proteins consisting of receptors, enzymes, transport proteins, nucleic acid binding proteins and extracellular matrix proteins.

11. The method of claim 10 wherein said receptors are selected from the group consisting of epidermal growth factor receptors, insulin receptors, platelet-derived growth factor receptors, tumor necrosis factor receptors, fibroblast growth factor receptors, erythropoietin receptor, lymphokine receptors and cytokine receptors.

12. The method of claim 1 wherein said allosteric protein is human epidermal growth factor.

13. Effector peptides produced by the method of claim 1.

14. A method of using effector peptides to alter a

functional activity of an allosteric protein comprising reacting said allosteric protein with one or more of said effector peptides, each of said effector peptides substantially identical to a region of a selected target sequence in the amino acid sequence of the protein, said sequence containing at least one site of intramolecular or intermolecular contact within an allosteric protein, said site involved in an allosteric transition altering the expression of functional activity of the allosteric protein.

15. The method of claim 14 wherein said effector peptides are produced by the method of claim 1.

16. The method of claim 14 wherein the amino acid sequence of the effector peptides is substantially identical to a sequence consisting of at least 3 amino acid residues in the amino acid sequence of the protein that participates in α-helix formation within the three-dimensional structure of the protein.

17. The method of claim 14 wherein the amino acid sequence of the effector peptides is substantially identical to a sequence of at least 3 amino acid residues in the amino acid sequence of the protein that participates in β-pleated sheet formation.

18. The method of claim 14 wherein the allosteric protein is selected from the group of proteins consisting of enzymes, transport proteins, nucleic acid binding proteins, receptor proteins and extracellular matrix proteins.

19. The method of claim 18 wherein said receptor proteins are selected from the group of proteins consisting of epidermal growth factor receptors, insulin receptors, platelet-derived growth factor receptors, tumor necrosis factor receptors, fibroblast growth factor receptors, erythropoietin receptor, lymphokine receptors and cytokine receptors.

20. The method of claim 14 wherein the allosteric protein is human epidermal growth factor receptor.

21. An effector peptide for inhibiting the tyrosine kinase activity of human epidermal growth factor receptor, said effector peptide substantially identical in sequence to a portion of the region between amino acids 646-1015 in the amino acid sequence encoding said receptor.

22. The peptide of claim 21 wherein said peptide is selected from the group consisting of (SEQ ID NO:6) V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, T-V-Q-L-I-T-Q-L-M-P, W-C-V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, V-K-I-T-D-F-G-L-A-K-L-L-G, M-A-L-E-S-I-L-H-R-I-Y-T, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, P-A-S-E-I-S-S-I-L-E-K, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, W-M-I-D-A-D-S-R-P-K-F, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, F-Y-R-A-L-M-D-E-E-D-M-D, D-D-V-V-D-A-D-E-Y-L-I-P, D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, A-K-L-L-G-A-E-E-K-E-Y-H-A-E-G, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, V-Q-I-A-K-G-M-N-Y-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, I-M-V-K-C-W-M-I-D-A-D, P-L-T-P-S-G-E-A-P, Y-L-V-I-Q-G-D and D-E-Y-L-I-P-Q-Q-G-F-F.

23. The peptide of claim 21 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) T-V-Q-L-I-T-Q-L-M-P, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, I-M-V-K-C-W-M-I-D-A-D, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, D-D-V-V-D-A-D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, Y-L-V-I-Q-G-D, and D-E-Y-L-I-P-Q-Q-G-F-F.

24. The peptide of claim 21 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D and I-M-V-K-C-W-M-I-D-A-D.

25. The peptide of claim 21 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D and V-Q-I-A-K-G-M-N-Y-L.

26. An effector peptide for stimulating the tyrosine kinase activity of human epidermal growth factor receptor by reaction of said peptide with the region between amino acids 646-1015 in the amino acid sequence encoding said receptor.

27. The peptide of claim 26 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) R-R-H-I-V-R-K-R-T and K-F-R-E-L-I-I-E-F-S-K-M-A-R-D.

28. The method of claim 14 wherein the allosteric protein is an enzyme and reaction of the effector peptide together with the allosteric protein increases the enzymatic activity of the allosteric protein.

29. The method of claim 14 wherein the allosteric protein is an enzyme and the incubation of the peptide together with the allosteric protein decreases the enzymatic activity of the allosteric protein.

30. The method of claim 14 wherein the allosteric protein is an enzyme and the activity of the allosteric protein is determined by measuring enzymatic activity.

31. A method for inhibiting the protein tyrosine kinase activity of human epidermal growth factor receptor comprising the steps of reacting an effector peptide that is at least 3 amino acids in length that has an amino acid sequence that is substantially identical to a portion of the amino acid sequence of human epidermal growth factor receptor occurring between amino acids 646-1015 of human epidermal growth factor receptor with human epidermal growth factor receptor to inhibit the protein tyrosine kinase activity of the human epidermal growth factor receptor.

32. The method of claim 31 wherein the effector peptide is selected from the group consisting of (SEQ ID NO:6) V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, T-V-Q-L-I-T-Q-L-M-P, W-C-V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, V-K-I-T-D-F-G-L-A-K-L-L-G, M-A-L-E-S-I-L-H-R-I-Y-T, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, P-A-S-E-I-S-S-I-L-E-K, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, W-M-I-D-A-D-S-R-P-K-F, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, F-Y-R-A-L-M-D-E-E-D-M-D, D-D-V-V-D-A-D-E-Y-L-I-P, D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, A-K-L-L-G-A-E-E-K-E-Y-H-A-E-G, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, V-Q-I-A-K-G-M-N-Y-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, I-M-V-K-C-W-M-I-D-A-D, P-L-Y-P-G-E-A-P, Y-L-V-I-Q-G-D and D-E-Y-L-I-P-Q-Q-G-F-F, when the peptide is present at a concentration of about 1 mM, causing at least 34% inhibition of the tyrosine kinase activity of human epidermal growth factor.

33. The method of claim 31 wherein the effector peptide is selected from the group consisting of (SEQ ID NO:6) T-V-Q-L-I-T-Q-L-M-P, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, I-M-V-K-C-W-M-I-D-A-D, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, D-D-V-V-D-A-D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, Y-L-V-I-Q-G-D, and D-E-Y-L-I-P-Q-Q-G-F-F when the peptide is present at a concentration of about 1 mM, causing at least 50% inhibition of the tyrosine kinase activity of human epidermal growth factor.

34. The method of claim 31 wherein the effector peptide is selected from the group consisting of (SEQ ID NO:6) K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D and I-M-V-K-C-W-M-I-D-A-D when the peptide is present at a concentration of about 1 mM causing at least 75% inhibition of the tyrosine kinase activity of human epidermal growth factor receptor.

35. The method of claim 31 wherein the effector peptide is selected from the group consisting of (SEQ ID NO:6) A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D and V-Q-I-A-K-G-M-N-Y-L, when the peptide is present at a concentration of about 1 mM causing at least 85% inhibition of the tyrosine kinase activity of human epidermal growth factor receptor.

36. The method of claim 31 wherein the effector peptide includes a region of the epidermal growth factor receptor that is involved in ATP binding.

37. A method of stimulating the tyrosine kinase activity of human epidermal growth factor receptor comprising the steps of reacting an effector peptide of at least 3 amino acids and having an amino acid sequence that is substantially identical to a portion of the amino acid sequence encoding human epidermal growth factor receptor, the peptide being selected from the group consisting of (SEQ ID NO:6) R-R-H-I-V-R-K-R-T and K-F-R-E-L-I-I-E-F-S-K-M-A-R-D with the human epidermal growth factor receptor to stimulate the tyrosine kinase activity of the human epidermal growth factor receptor.

38. An effector peptide of at least 3 amino acids in length and substantially identical to a region of a selected target sequence in the amino acid sequence of an allosteric protein, said sequence containing at least one site of intramolecular or intermolecular contact within the allosteric protein, said site involved in an allosteric transition altering the expression of functional activity of the allosteric protein, and said peptide causing inhibition or activation of a biological activity of said allosteric protein when reacted with the protein.

39. An effector peptide that substantially inhibits the tyrosine kinase activity of epidermal growth factor receptor when reacted with the receptor, the peptide being substantially identical to a portion of the amino acid sequence encoding the

receptor occurring between amino acids 646-1015 of the receptor.

40. The effector peptide of claim 39 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, T-V-Q-L-I-T-Q-L-M-P, W-C-V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, V-K-I-T-D-F-G-L-A-K-L-L-G, M-A-L-E-S-I-L-H-R-I-Y-T, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, P-A-S-E-I-S-S-I-L-E-K, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, W-M-I-D-A-D-S-R-P-K-F, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, F-Y-R-A-L-M-D-E-E-D-M-D, D-D-V-V-D-A-D-E-Y-L-I-P, D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, A-K-L-L-G-A-E-E-K-E-Y-H-A-E-G, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, V-Q-I-A-K-G-M-N-Y-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, I-M-V-K-C-W-M-I-D-A-D, P-L-T-P-S-G-E-A-P, Y-L-V-I-Q-G-D and D-E-Y-L-I-P-Q-Q-G-F-F.

41. The effector peptide of claim 39 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) T-V-Q-L-I-T-Q-L-M-P, K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, C-L-L-D-Y-V-R-E, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, P-I-C-T-I-D-V-Y-M-I-M-V-K-C, I-M-V-K-C-W-M-I-D-A-D, Q-S-D-V-W-S-Y-G-V-T-V-W-E-L-M, V-L-G-S-G-A-F-G-T-V-Y-K-G-L-W, K-F-R-E-L-I-I-E-F-S-K-M-A-R-D, D-D-V-V-D-A-D-E-Y-L-I-P, N-Q-A-L-L-R-I-L-K-E-T-E-F-K-K, T-E-F-K-K-I-K-V-L-G-S-G-A, Y-L-V-I-Q-G-D, and D-E-Y-L-I-P-Q-Q-G-F-F.

42. The effector peptide of claim 39 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) K-V-K-I-P-V-A-I, I-T-Q-L-M-P-F-G-C-L-L-D, W-C-V-Q-I-A-K-G-M-N-Y-L, V-Q-I-A-K-G-M-N-Y-L, G-M-N-Y-L-E-D-R-R-L-V-H-R-D-L, A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D and I-M-V-K-C-W-M-I-D-A-D.

43. The effector peptide of claim 39 selected from the group consisting of (SEQ ID NO:6) A-A-R-N-V-L-V-K-T-P-Q-H-V-K-I-T, Y-L-V-I-Q-G-D and V-Q-I-A-K-G-M-N-Y-L.

44. An effector peptide that activates the tyrosine kinase activity of epidermal growth factor receptor when reacted with the receptor, the peptide being substantially identical to a portion of the amino acid sequence encoding the receptor between amino acid residues 646-950.

45. The effector peptide of claim 44 wherein the peptide is selected from the group consisting of (SEQ ID NO:6) R-R-H-I-V-R-K-R-T and K-F-R-E-L-I-I-E-F-S-K-M-A-R-D.

46. The effector peptide of claim 44 wherein the peptide is (SEQ ID NO:6) K-F-R-E-L-I-I-E-F-S-K-M-A-R-D.

RECEPTOR COMPARISON

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INS-R  851  RLRQPDGPLGPLYASSNPEYLSASDVFPCSVYVPDEWEVSREKITLLR
PDGF-R 525  QKKPRYEIRWKVIESVSSDGHEYIYVDPVQLPYDSTWELPRDQLVLGR
HER-1  646  RRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRILKETEFKKIK
HER-2  678  RRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVK
            ——42——                  ——13——————18————
                                                ——45

INS-R  899  ELGQGSFGMVYEGNARDIIKGEAETRVAVKTVNESASLRERIE-FLNE
PDGF-R 573  TLGSGAFGQVVEGTAHGLSHSQATMKVAVKMLK-STARSSEKQALMSE
HER-1  694  VLGSGAFGTVYKGLWIPEGE-KVKIPVAIKELREATSPKANKE-ILDE
HER-2  726  VLGSGAFGTVYKGIWIPDGE-NVKIPVAIKVLRENTSPKANKE-ILDE
            ————49————      ——32——

INS-R 1036  ASVMKGFTCHH--VVRLLGVVSKGQPTLVVMELMAHGDLKSYLRSLRPE
PDGF-R 620  LKIM-SHLGPHLNVVNLLGACTKGGPIYIITEYCRYGDLVDYLHR----
HER-1  741  AYVMASVDNPH--VCRLLGICLTST-VQLITQLMPFGCLLDYVR-EHKD
HER-2  773  AYVMAGVGSPY--VSRLLGICLTST-VQLVTQLMPYGCLLDHVR-ENRG
                                  ——14——23——28—

INS-R 1081  AENNPGRPPPTLQEMIQMAAEIADGMAYLNAKKFVHRDLAARNCMVAH
PDGF-R 770  ----------DLVGFSYQVANGMDFLASKNCVHRDLAARNVLIGE
HER-1  785  NI--------GSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKT
HER-2  809  RL--------GSQYLLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKS
                          ——5——6————————43

INS-R 1131  DFTVKIGDFGMTRDI--YETDYYRKGGKGLLPVRWMAPESLKDGVFTT
PDGF-R 812  GKLVKICDFGLARDIM-RDSNYISKGS-TYLPLKWMAPESIFNSLYTT
HER-1  826  PQHVKITDFGLAKLLGAEEKEYHAEGG--KVPIKWMALESILHRIYTH
HER-2  858  PNHVKITDFGLARLLDIDETEYHADGG--KVPIKWMALESILRRRFTH
            43——10——11————————44——

INS-R 1177  SSDMWSFGVVLWEITSLAEQPYQGLSNEQVLKFVMDGGY-LDQPDNCP
PDGF-R 851  LSDVWSFGILLWEIFTLGGTPYPELPMNDQFYNAIKRGYRMAQPAHAS
HER-1  872  QSDVWSYGVTVWELMTFGSKPYDGIPASEISSIL-EKGERLPQPPICT
HER-2  904  QSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLL-EKGERLPQPPICT
            ————50————————21————————

INS-R 1224  ERVTDLMRMCWQFNPNMRPTFLEIVNLLKDDLHPSFPEVSFFHSEENK
PDGF-R 899  DEIYEIMQKCWEEKFETRPPFSQLVLLLERLLGEGYKKKYQQVDEEFL
HER-1  920  IDVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGDERMH
HER-2  952  IDVYMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGP
            ——48——39————26——
                ——25——

INS-R 1272  APESEELEMEFENMENVPLDRSSHCQREEAGGRDGGSSLGFKRSYEEH
PDGF-R 947  RSDHPAILRSQARFPGIHSLRSPLDTSSVLYTAVQPNESDNDYIIPLP
HER-1  966  LPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLS
HER-2  998  A-SPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFF
                ——27——————17————
                              ——41——
                              -41A-

INS-R 1320  IPYTHMNGGLLNGRILTLPRSNPS
PDGF-R 995  DPKPDVADEGLPEGSPSLASSTLNEVNTSSTISCDSPLELQEEP
HER-1 1014  SLSATSNNSTVVACIDRNGLQSCPIKEDSFLQRYSSDDPTGALTEDSIDD
```

FIG. 1

NO.	PEPTIDE		MW	% INHIBITION AT 1mM EGF−	% INHIBITION AT 1mM EGF+	COMP TYPE	ED50(mM) EGF−	ED50(mM) EGF+
42	646−654	RRHIVRKRT	1261	0	0			
1	650−659	VRKRTLRRLL	1350	0	0			
12	655−667	LRRLLQERELVEP	1690	0	0			
12V1		LRRLLQEREKVEP	1705					
12A		QERELVEP	1039					
12B	660−667	ELVEP	937					
12C	663−667	TLRRLL	1068					
13	654−659	PLTPSGEAP	908	37	48		1.0	1.0
13A	667−675	LTPSG	457					
2	668−672	GEAPNQALL	952	22	13			
18	672−680	NQALLRILKETEFKK	1870	50	33		P	P
45	676−690	TEFKKIKVLGSGA	1417	50	40		P	P
49	686−698	VLGSGAFGTVYKGLW	1594	53	68		0.4	0.4
9	693−707	KGLWIPEG	939	71	34			
19	704−711	IPEGEKVK	939	0	0			
32	718−715	KVKIPVAI	907	70	81	C/NC	0.3	0.2
32A	713−720	KVKIP	565	0	0			
32B	713−717	VKIP	496	0	14			
36	714−717	IPVAIKELREATS	1466	0	0			
36A	716−721	IPVAIK	621	0	10			>0.5
22	724−733	REATSPKANK	1141	59	2			
46	729−743	PKANKEILDEAYVMA	1731	67	24			>2.0
47	741−751	VMASVDNPHVC	1260	48	8			
3	759−764	HVCRLLGICLTSTVQL	1794	INSOLUBLE				
3A	760−765	STVQLI	700	39	19			
3B	761−766	TVQLIT	714	0	0			
14	761−770	TVQLITQLMP	1183	0	51		P	P
23	765−776	ITQLMPFGCLLD	1390	92	81		n.a.	0.2
28	773−780	CLLDYVRE	1050	62	52		1.0	1.0
33	777−785	YVREHKDNI	1213	49	10			

FIG. 2a

STIMULATION AT 1mM		STRUCTURE	STRUCTURE
EGF−	EGF+	PEPTIDE ANALYSIS	EGF−R ANALYSIS
2.05	2.94	444411111	223222222
1.3	1.34@0.25	2222221111	2222244441
0	0	4444111111133	44441111111113
0	0	334444333	334444344
0	0	334444111	434444111
0	0	1111111111111111	
0	0	111113333333	1111111111144
0	0	44442222222222	111144443344444
0	0	33333333	
0	0	44441111	
0	0	33111111	33111111
0	0	33333	33111
0	0	3333	3111
1.08	1.3	33111111111111	
0	0	333333	111111
0	0	3344441111	
0	0	44441111112222	
0	0	11111133333	
0	0	222222	222222
0	0	222222	222222
0	0	2222223333	2222223344
0	0	222222222222	
0	0	22222211	
0	0	111111111	

FIG. 2b

				42	2					
4	782–790	KDNIGSQYL	1077	PROBLEM IN SYNTHESIS						
34	786–797	GSQYLLNWCVQI	1463	56.3@0.31	77.7@0.31					
5	793–804	WCVQIAKGMNYL	1465	83	93			0.1	0.1	
5A	795–804	VQIAKGMNYL	1176					0.2	0.2	
6A	799–804	KGMNYL	765							
6	800–814	GMNYLEDRRLVHRDL	1926	73	81			0.5	0.3	
6B	800–805	GMNYLE		53	51					
7	810–820	VHRDLAARNVL	1303	36	0					
43	815–830	AARNVLVKTPQHVKIT	1863	65	89	NC		0.4	0.1	
43V1		AARNVLVKTPQ–VKIT	1726							
43A	827–830	VKIT	500	17	36					
43B	826–830	HVKIT	637	19	0					
43C	A825–830	Q–VKIT	628	47	25					
43D	825–830	QHVKIT	765	44	19					
43E	822–830	KTPQHVKIT	1091	0	39			P	P	
10	827–839	VKITDFGLAKLLG	1414	0	44					
10V1		VKITDFGKAKKKG								
10A	827–833	VKITDGF								
10B	831–836	DFGLAK	690					P	P	
11	835–849	AKLLGAEEKEYHAEG	1684	13	34					
20	846–855	HAEGGKVPIK	1075	48	0					
37	850–861	GKVPIKWMALES	1398	26	14					
44	857–868	MALESILHRIYT	1535	26	48					
44A	857–862	MALESI	703	14	24					
38	864–875	HRIYTHQSDVWS	1568	42	0					
50	870–884	QSDVWSYGVTVWELM	1839	12	55					
50A	873–882	VWSYGVTVWE	1265	INSOLUBLE						
15	880–890	VWELMTFGSKP	1334							
29	885–898	TFGSKPYDGIPASE	1508	3	16					
21	895–905	PASEISSILEK	1213	39	39	NC		>1.0	>1.0	
16	899–913	ISSILEKGERLPQPP	1703	12	13					

FIG. 2c

0	0	4444222222	
0	0	222222322222	222111111133
0	0	1111112222	2111111334
0	0	444434444111111	111334444111111
0	0	444411	
0	0	11111111111	
0	0	111113344442222	144443344441111
0	0	2222	1111
0	1.1@0.25	22222	41111
0	0		
0	0	222222	441111
0	0	344442222	344441111
0	0	1111111111111	1111111111111
0	0	111111111111111	111111111111444
0	0	4444333333	
0	0	3111111111111	
0	0	111111222222	111122222244
0	0	444422	111122
0	0	222222444422	
0	0	44444442222221	433344442222221
0	0	4444444433333	
0	0	4441111111	4441111111
0	0	111111144443330	

FIG. 2d

24	908-918	RLPQPPICTID	1292	0	4			0.6
48	913-926	PICTIDVYMIMVKC	1668	n.d.	58@0.625			
48A	916-924	TIDVYMIMV	1138	n.d.	39			<0.2
25	922-932	IMVKCWMIDAD	1364		78		>1.0	>1.0
39	927-937	WMIDADSRPKF	1405	31	42			
39A	930-937	DADSRPKF	975	0	0			
39B	936-942	KFRELII	958	0	0			
35	930-942	DADSRPKFRELII	1755	0	0			
35A	930-936	DADSRPK	828					
26	936-950	KFRELIIEFSKMARD	2078	73	54		0.3	0.5
26A	936-945	KFRELIIEFS	1321	INSOLUBLE				
51	944-956	FSKMARDPQRYLV	1650	55	20			
30	951-961	PQRYLVIQGDE	1202	11	24		P	P
30A	954-960	YLVIQGD	847	99	98		0.3	0.1
40	961-973	ERMHLPSPTDSNF	1619		30			>1.0
8	968-977	PTDSNFYRAL	1223	0	15			
27	973-985	FYRALMDEEDMD	1689	20	46		>1.0	1.0
27A	975-982	RALMDEED	1018	0	0			
31	979-991	DEEDMDDVVDADE	1536	19	28			
17	984-995	DDVVDADEYLIP	1403	47	59		0.5	0.6
17A	987-992	VDADEY	765	n.d.	29			>1.0
41	990-1000	DEYLIPQQGFF	1396	38	59		1.0	0.7
41A	990-995	DEYLIP	789	79	73	NC	0.5	0.5
41B	990-995	DEFLIP	773	7	4			
41C	991-995	EFLIP	674	0	0			
41D	995-1000	PQQGFF	763	11	15			
41E	A990-995	RRDEYLIP	1101	12	32		0.0	0.5
52	995-1005	PQQGFFSSPST	1222	INSOLUBLE				
53	1001-1015	SSPSTSRTPLLSSLS	1403	0	0			

FIG. 2e

1.1	0	33334444222	
0	0	22222222222222	44422222222444
0	1.6@.25	222222222	222222224
0	0		
0	0	11111113333	41111114444
1.06@0.5	1.2@0.5	44443333	11114444
0.0@0.5	1.2@0.5	1111111	4411111
0	0	4444444411111	1111444411111
11.5@0.062	5.4@0.062	111111111111111	4411111111111444
0	0	1111114444222	
0	0	4444111111	42222224444
0	0	2222221	2222444
0	0		
0	0	4444444411	
0	0	111111111111	4411111114444
0	1.21@0.5	11111111	11111114
0	0	1111111111111	
0	0	11111113333	4411111112222
0	0	111111	111112
0	0	33222222222	11222222334
0	0	333333	112222
0	0	333333	
0	0	33333	
0	0	444422	
0	0		
0	0	44444443333333	

n.d., none detected; n.a., not assayed
1=Alpha Helix; 2=Random Coil;
3=Beta Turn; 4=Beta Sheet
C=Competitive Inhibition;
NC=Noncompetitive Inhibition

FIG. 2f

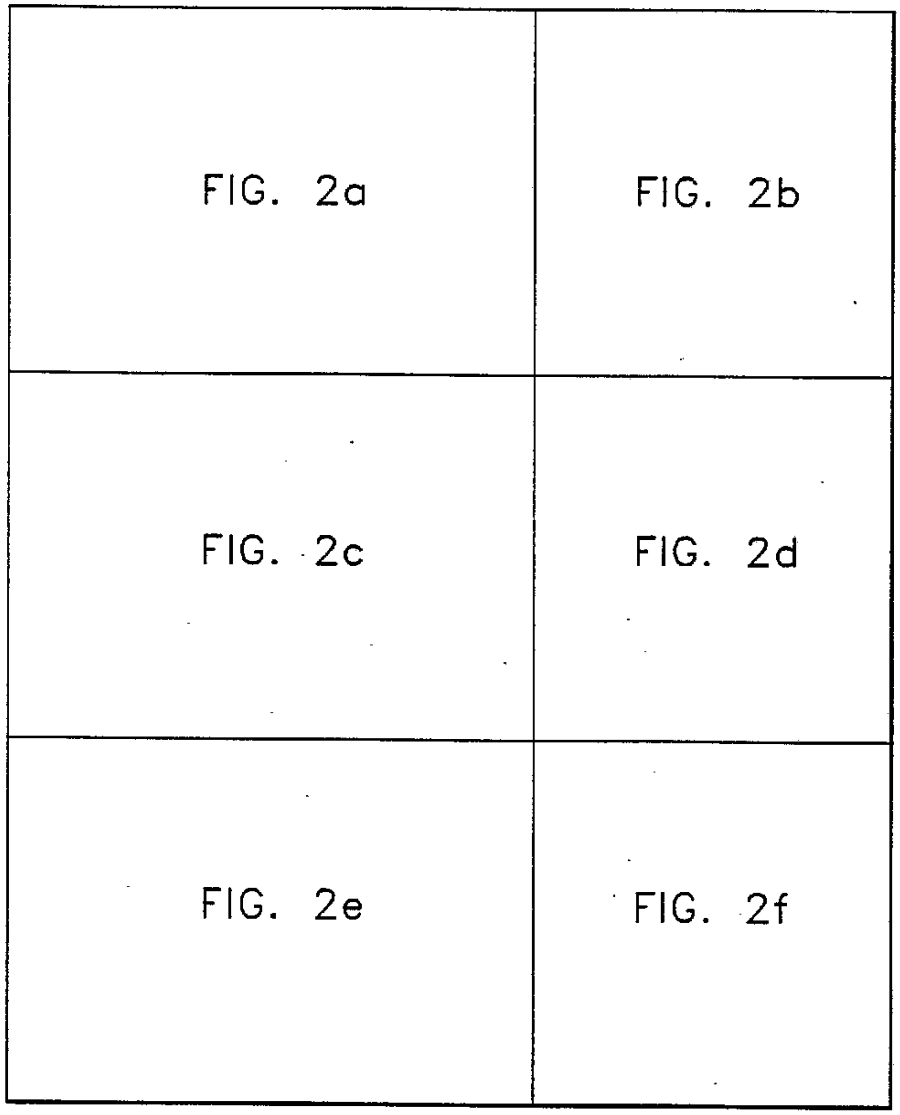


FIG. 2g

646 730

RRHIVRKRT NQALLRILKETEFKK VLGSGAGTVYKGLW KVKIPVAI REATSPK

LRRLLQERELVEP GEAPNQALL TEFKKIKVLGSGA KGLWIPEG IPVAIKELREATS

VRKRTLRRLL PLTPSGEAP IPEGEKVK PK

731 820

ANK HVCRLLGICLTSTVQL YVREHKDNI WCVQIAKGMNYL VHRDLAARNVL

VMASVDNPHVC ITQLMPFGCLLD KDNIGSQYL GMNYLEDRRLVHRDL

ANKEILDEAYVMA TVQLITQLMP CLLDYVRE AARNVL

821 910

VKITDFGLAKLLG HAEGGKVPIK MALESILHRIYT TFGSKPYDGIPASE

AKLLGAEEKEYHAEG HRIYTHQSDVWS VWELMTFGSKP PASEISSILEK RLP

VKTPQHVKIT GKVPIKWMALES QSDVWSYGVTVWELM ISSILEKGERLP

911 1000

PICTIDVYMIMVKC KFRELIIEFSKMARD PTDSNFYRAL DDVVDADEYLIP

QPPICTID WMIDADSRPKF FSKMARDPQRYLV ERMHLPSPTDSNF DEEDMDDVVDADE

QPP DADSRPKFRELII PQRYLVIQGDE FYRALMDEEDMD DEYLIPQQGFF

IMVKCWMIDAD

FIG. 3

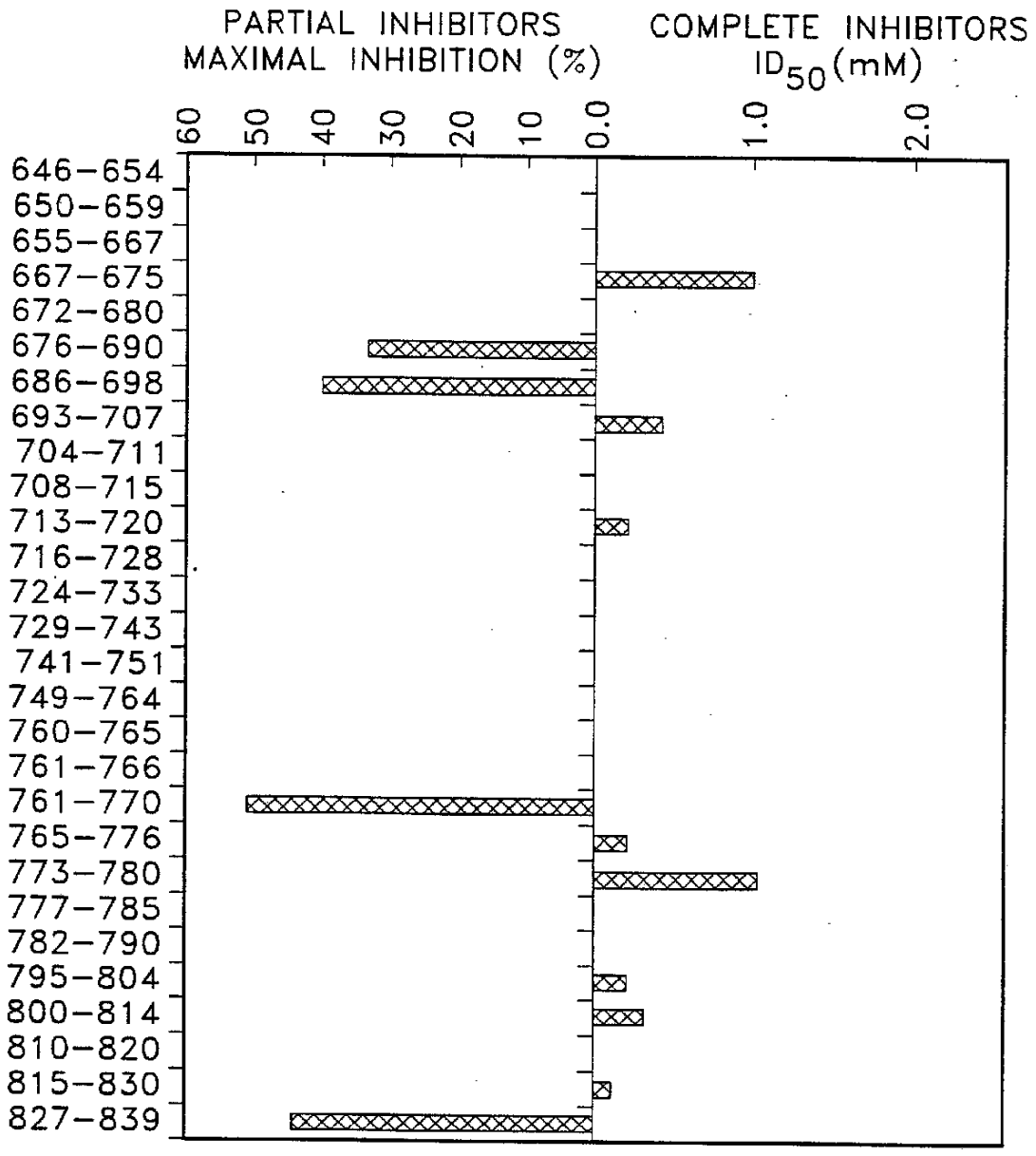

PARTIAL INHIBITORS
MAXIMAL INHIBITION (%)
COMPLETE INHIBITORS
ID_{50}(mM)
60
50
40
30
20
10
0.0
1.0
2.0
646–654
650–659
655–667
667–675
672–680
676–690
686–698
693–707
704–711
708–715
713–720
716–728
724–733
729–743
741–751
749–764
760–765
761–766
761–770
765–776
773–780
777–785
782–790
795–804
800–814
810–820
815–830
827–839

FIG. 4a

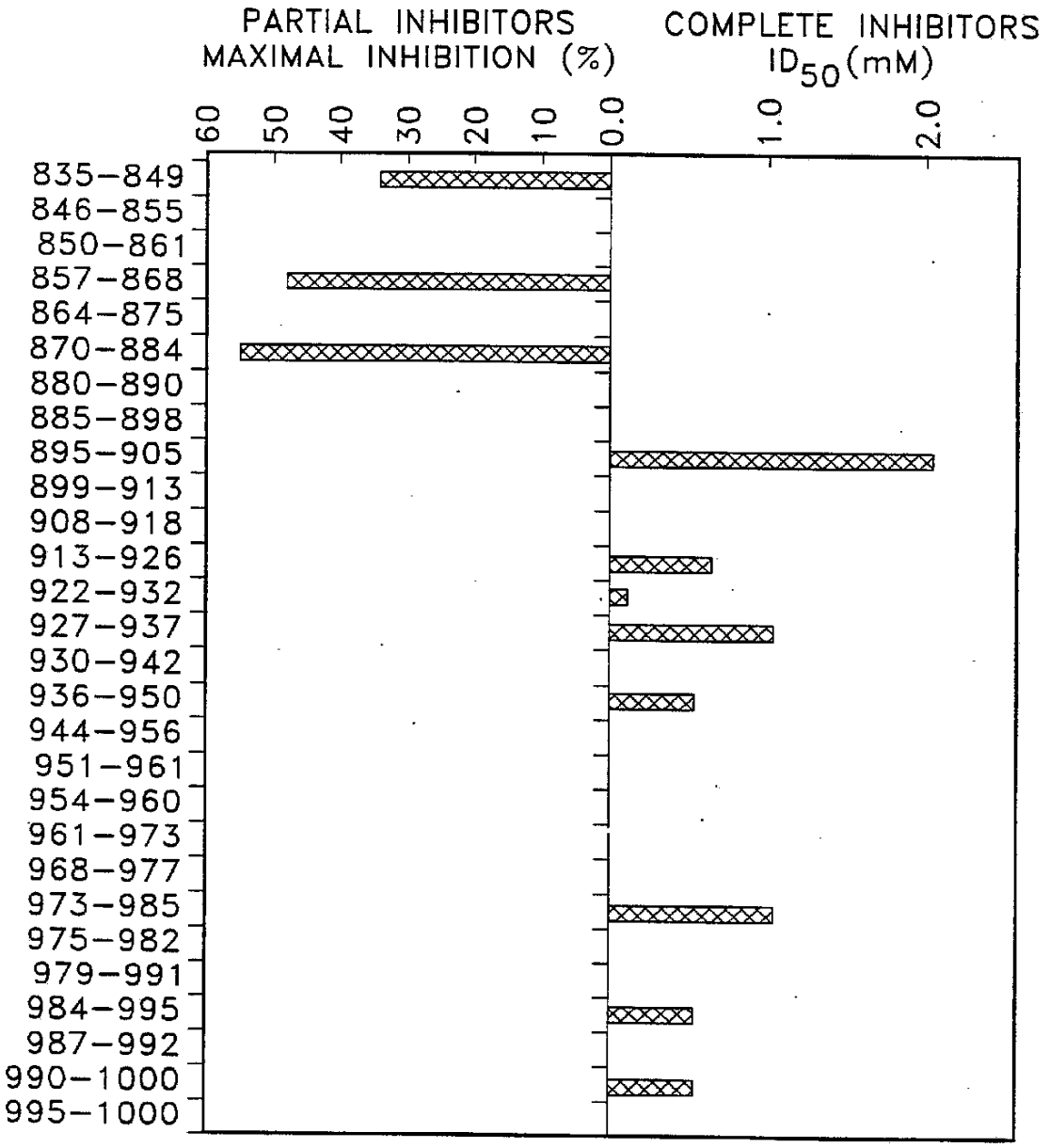

PARTIAL INHIBITORS
MAXIMAL INHIBITION (%)
COMPLETE INHIBITORS
ID50 (mM)
60
50
40
30
20
10
0.0
1.0
2.0
835–849
846–855
850–861
857–868
864–875
870–884
880–890
885–898
895–905
899–913
908–918
913–926
922–932
927–937
930–942
936–950
944–956
951–961
954–960
961–973
968–977
973–985
975–982
979–991
984–995
987–992
990–1000
995–1000

FIG. 4b

NO.	PEPTIDE	SEQUENCE		% INHIBITION AT 1mM		COMP	ED50(mM)		STRUCTURE	STRUCTURE
			MW	EGF−	EGF+	TYPE	EGF−	EGF+	PEPTIDE ANALYSIS	EGF−R ANALYSIS
13	667−675	PLTPSGEAP	908	37	48		1.0	1.0	334444333	334444344
49	693−707	VLGSGAFGTVYKGLW	1594	53	68		0.4	0.4	44442222222222	111144443344444
32	713−720	KVKIPVAI	907	70	81	C/NC	0.3	0.2	33111111	33111111
23	765−776	ITQLMPFGCLLD	1390	92	81		n.a.	0.2	222222222222	
28	773−780	CLLDYVRE	1050	62	52	NC	1.0	1.0	22222211	
5	793−804	WCVQIAKGMNYL	1465	56@	78@		0.1	0.1	222222322222	222111111133
5A	795−804	VQIAKGMNYL	1176	83	93		0.2	0.2	1111112222	2111111334
6	800−814	GMNYLEDRRLVHRDL	1926	73	81		0.5	0.3	4444344444111111	1113344441111111
43	815−830	AARNVLVKTPQHVKIT	1863	65	89	NC	0.4	0.1	111113344442222	14444333444411111
21	895−905	PASEISSILEK	1213	39	35	NC	>1.0	2.0	4441111111	4441111111
48	913−926	PICTIDVYMIMVKC	1668	n.d.	58#			0.6	22222222222222	44422222222444
25	922−932	IMVKCWMIDAD	1364	n.a.	75		n.a.	0.2	22222211111	
39	927−937	WMIDADSRPKF	1405	31	42		>1.0	>1.0	11111113333	41111114444
26	936−950	KFRELIIEFSKMARD	2078	73	54		0.3	0.5	111111111111111	4411111111111444
27	973−985	FYRALMDEEDMD	1689	20	46	*	>1.0	1.0	111111111111	4411111114444
17	984−995	DDVVDADEYLIP	1403	47	59		0.5	0.6	11111113333	4411111112222
41	990−1000	DEYLIPQQGFF	1396	38	59		>1.0	0.7	33222222222	11222222334

n.d.=none detected; n.a.=not assayed
C=Competitive Inhibition; NC=Noncompetitive
@=Inhibition at 0.3mM
#=Inhibition at 0.6mM
*=peptide 27 decreased the Km for substrate to one−third while inhibiting by approximately 50%

1=Alpha Helix; 2=Random Coil
3=Beta Turn; 4=Beta Sheet

FIG. 5a

NO.	PEPTIDE	SEQUENCE	MW	%INHIBITION AT 1mM		STRUCTURE	STRUCTURE
				EGF−	EGF+	PEPTIDE ANALYSIS	EGF−R ANALYSIS
18	676−690	NQALLRILKETEFKK	1870	50	33	111111111111111	
45	686−698	TEFKKIKVLGSGA	1417	50	40	111113333333	1111111111144
14	761−770	TVQLITQLMP	1183	0	51	2222223333	2222223344
10	827−839	VKITDFGLAKLLG	1414	0	44	1111111111111	1111111111111
11	835−849	AKLLGAEEKEYHAEG	1684	13	34	111111111111111	111111111111444
44	857−868	MALESILHRIYT	1535	26	48	111111222222	111122222244
50	870−884	QSDVWSYGVTVWELM	1839	12	55	44444442222221	433344442222221

FIG. 6

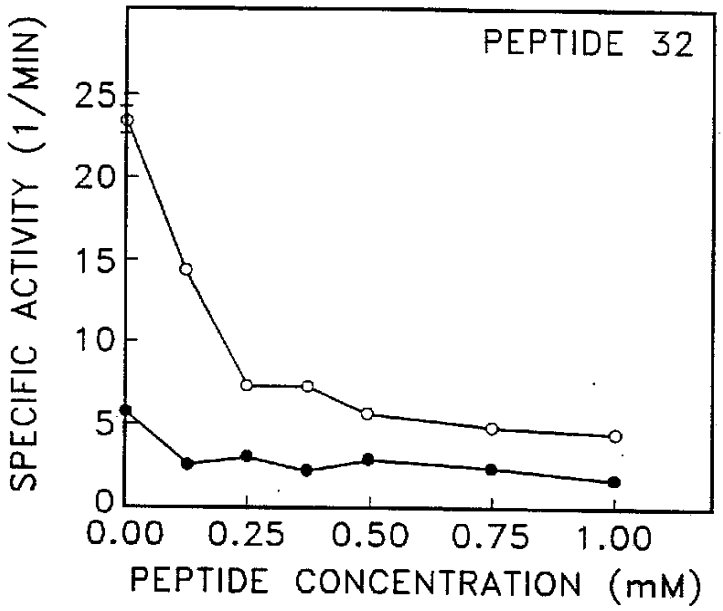

PEPTIDE 32
SPECIFIC ACTIVITY (1/MIN)
25
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
PEPTIDE CONCENTRATION (mM)

FIG. 7a

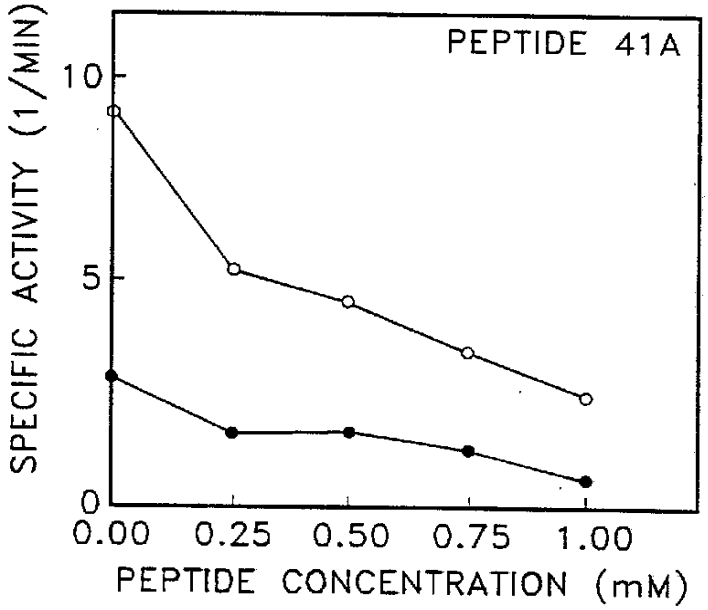

PEPTIDE 41A
SPECIFIC ACTIVITY (1/MIN)
10
5
0
0.00
0.25
0.50
0.75
1.00
PEPTIDE CONCENTRATION (mM)

FIG. 7b

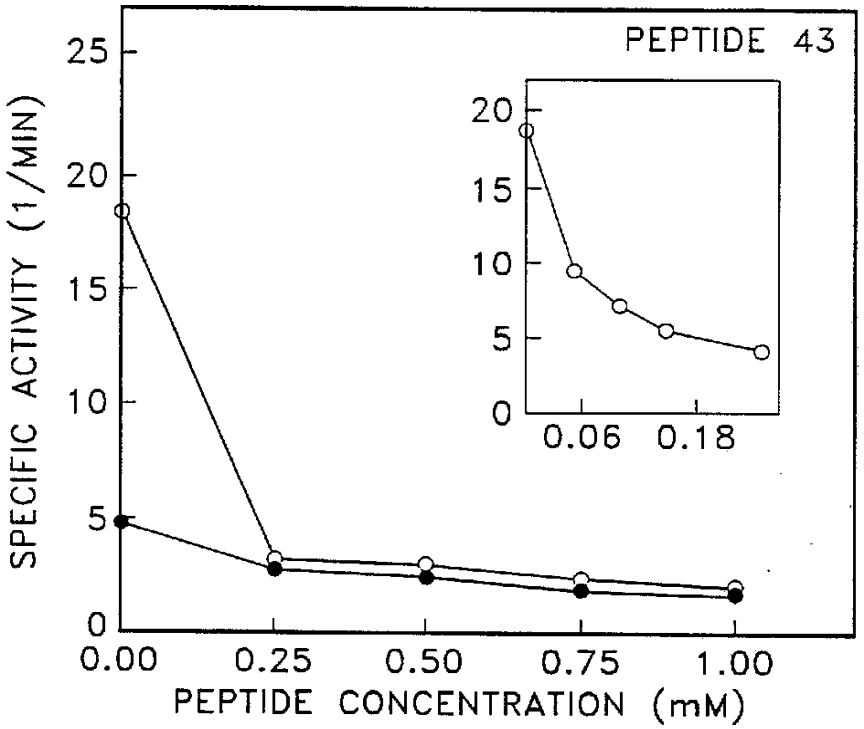

PEPTIDE 43
SPECIFIC ACTIVITY (1/MIN)
PEPTIDE CONCENTRATION (mM)
0.00
0.25
0.50
0.75
1.00
0.06
0.18

FIG. 7c

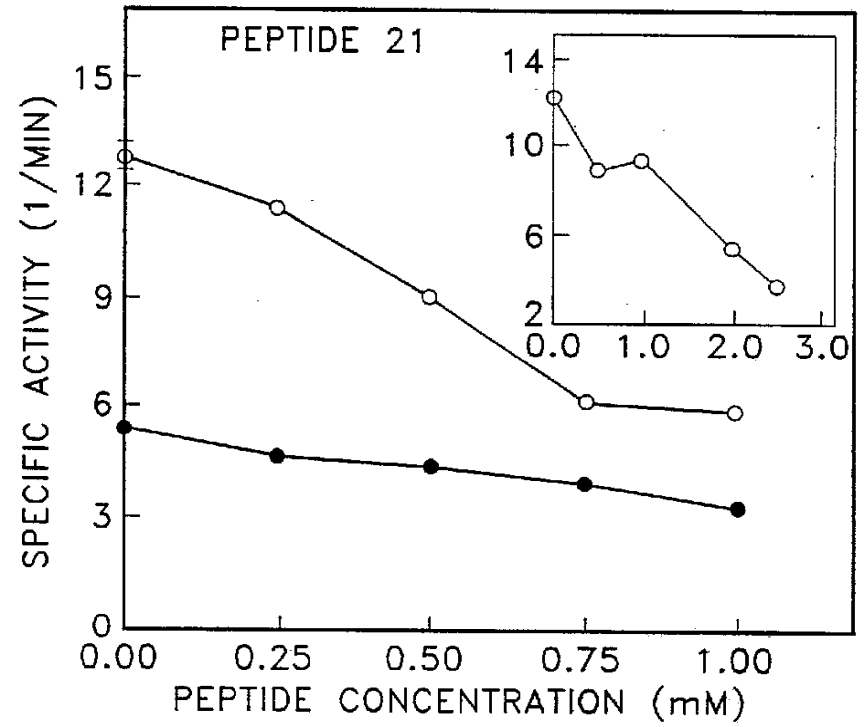

PEPTIDE 21
SPECIFIC ACTIVITY (1/MIN)
PEPTIDE CONCENTRATION (mM)
0.00
0.25
0.50
0.75
1.00
0.0
1.0
2.0
3.0

FIG. 7d

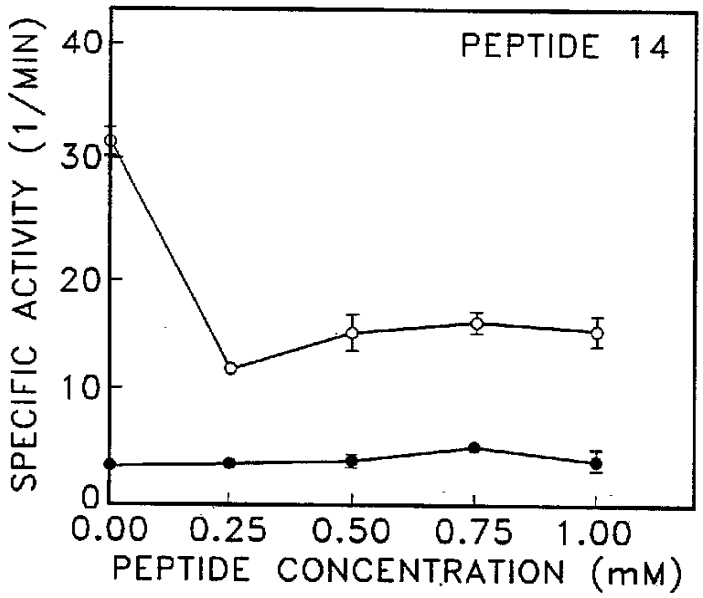
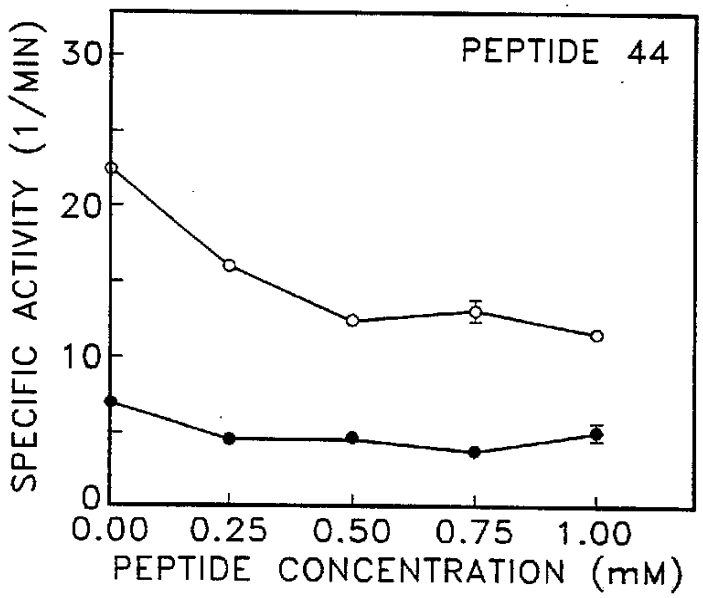

PEPTIDE 14
SPECIFIC ACTIVITY (1/MIN)
40
30
20
10
0
0.00 0.25 0.50 0.75 1.00
PEPTIDE CONCENTRATION (mM)

FIG. 8a


PEPTIDE 44
SPECIFIC ACTIVITY (1/MIN)
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20
10
0
0.00 0.25 0.50 0.75 1.00
PEPTIDE CONCENTRATION (mM)

FIG. 8b

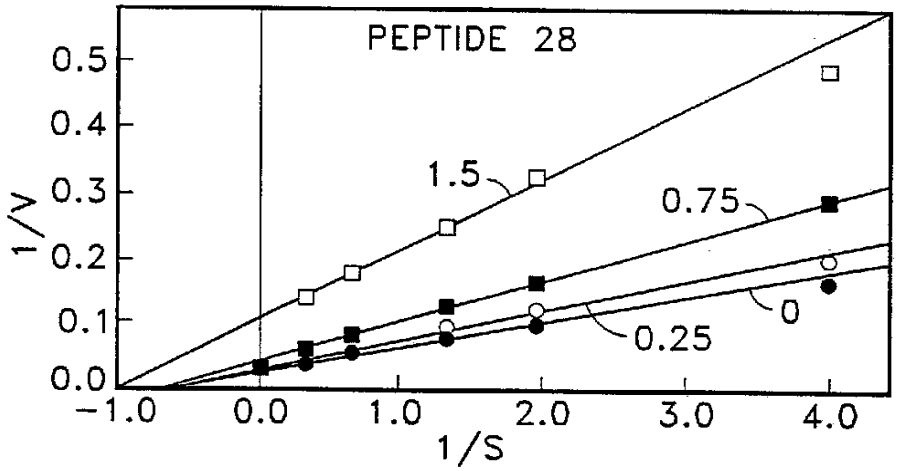

FIG. 9a

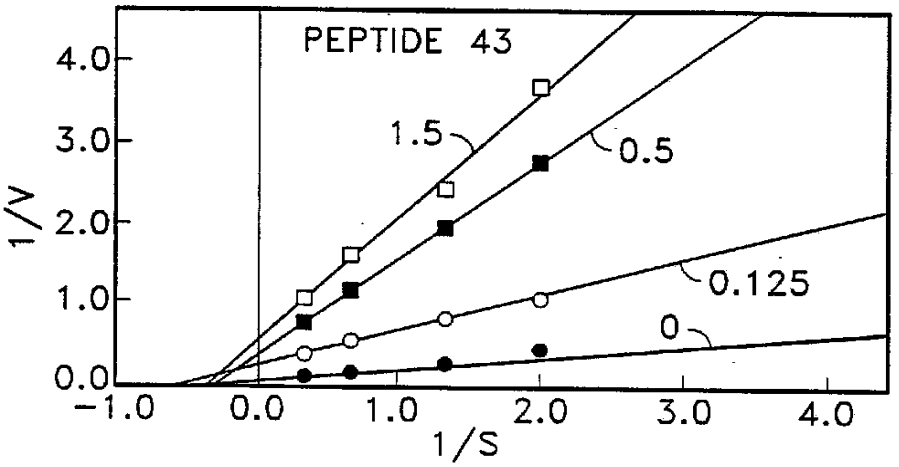

FIG. 9b

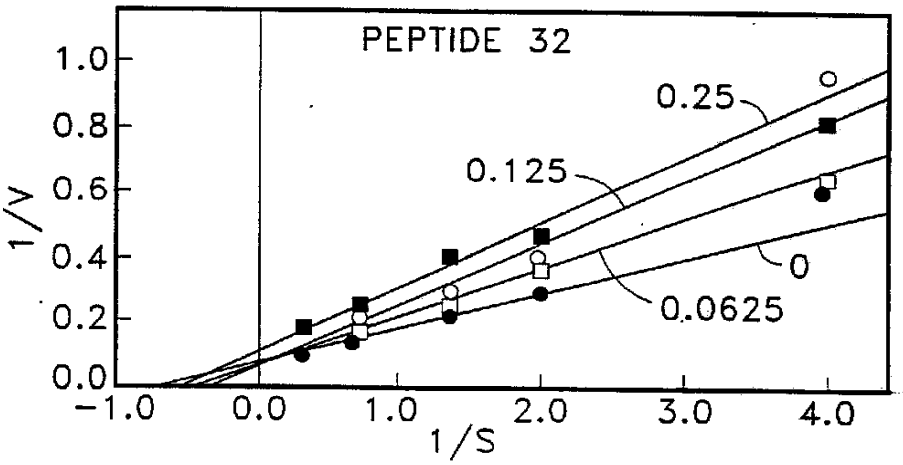

FIG. 9c

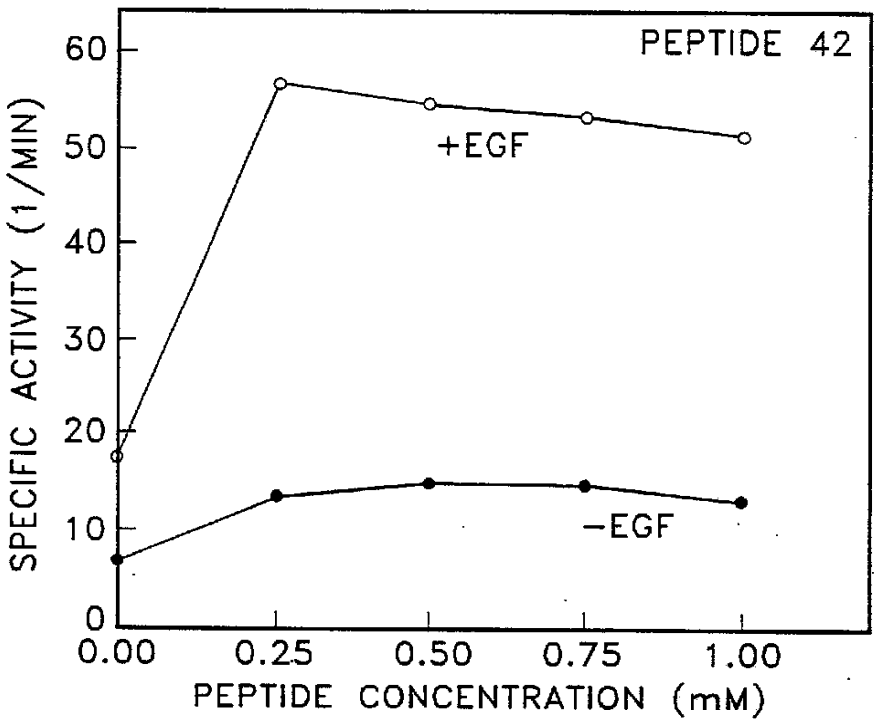

FIG. 10a

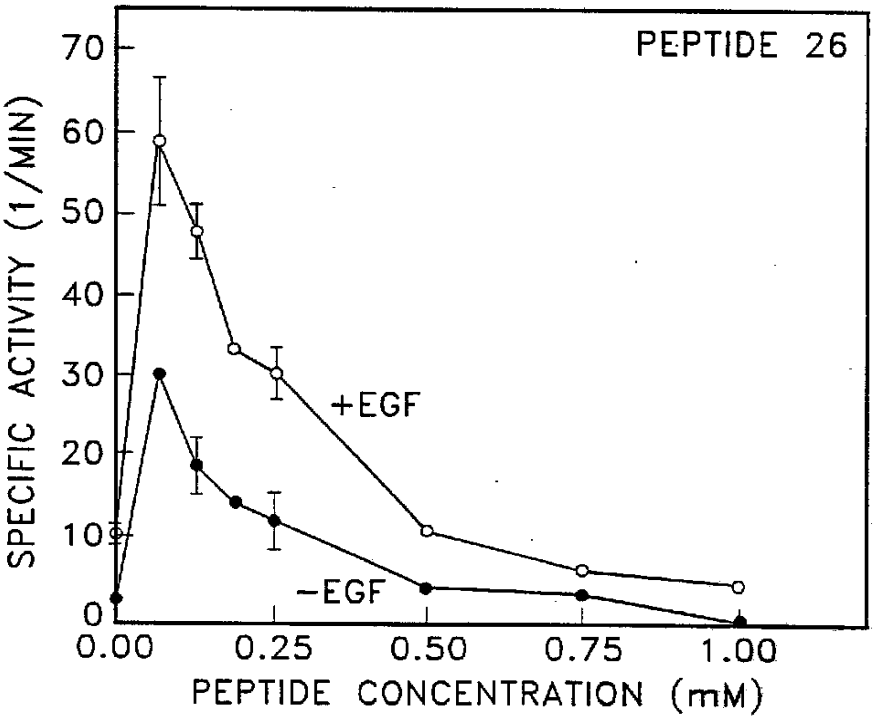

FIG. 10b

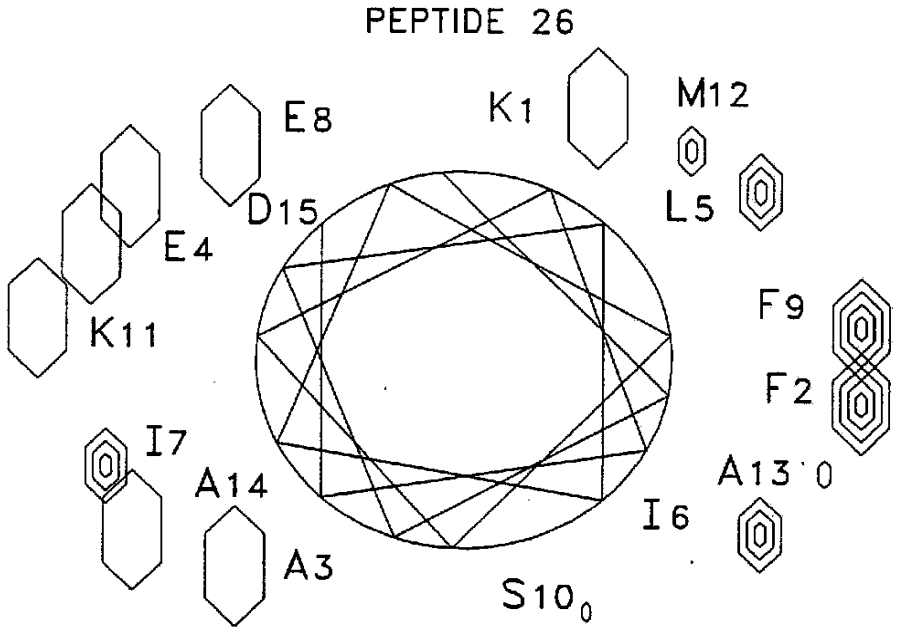

FIG. 11a

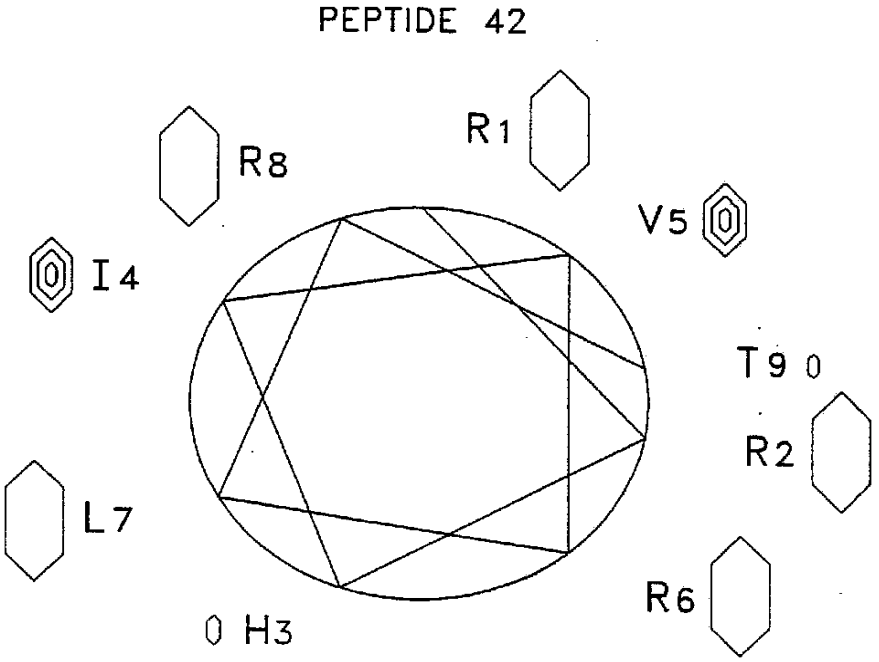

FIG. 11b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00581

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02; C07K 5/00, 7/00; G01N 33/566
US CL :436/501; 424/88; 530/326

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/501; 424/88; 530/326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: EGF receptor, nested or overlapping fragments, epitope mapping

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,933,294 (Waterfield et al.) 12 June 1990, entire document, especially column 2, line 55 to col. 3, line 2, also column 3, lines 31-33.	1-46
Y	US, A, 5,079,228 (Cohen et al.) 07 January 1992, col.3, lines 30-45.	1-12
Y	J. Immunological Methods, volume 122, issued 1989, S. Demotz et al., "A novel and simple procedure for determining T cell epitopes in protein antigens", pages 67-72, entire document.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 1993	29 MAR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer LORRAINE M. SPECTOR, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★